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OIPE JC34 APR 0 8 2004 PATENT & TRADEMARK OFFICE

FEE TRANSMITTAL

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT $330

Complete if Known	
Application Number	09/469,485
Filing Date	December 22, 1999
First Named Inventor	Qinjian Zhao
Examiner Name	Shanon A. Foley
Group Art Unit	1648
Attorney Docket Number	20369Y

METHOD OF PAYMENT

☒ Deposit Account

Deposit Account Number: 13-2755

Deposit Account Name: Merck & Co., Inc.

The Director is authorized to:

☒ Charge fee(s) indicated below ☒ Credit any overpayments

☒ Charge any additional fee(s) during the pendency of this application

FEE CALCULATION

1. BASIC FILING FEE

Large Fee Code	Entity Fee ($)	Fee Description	Fee Paid
1001	770	Utility filing fee	
1002	340	Design filing fee	
1004	770	Reissue filing fee	
1005	160	Provisional filing fee	

SUBTOTAL(1) $0

2. EXTRA CLAIM FEES

				Extra		Fee from below	Fee Paid
Total Claims		-	20 ** =	0	x	$18 =	0
Independent Claims		-	3 ** =	0	x	$86 =	0
Multiple Dependent Claims						$290 =	

***or number previously paid, if greater; For Reissues, see below*

Large Fee Code	Entity Fee ($)	Fee Description
1202	18	Claims in excess of 20
1201	86	Independent claims in excess of 3
1203	290	Multiple dependent claim, if not paid
1204	86	**Reissue independent claims over original patent
1205	18	**Reissue claims in excess of 20 and over original patent

SUBTOTAL(2) $0

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Fee Code	Large Entity Fee ($)	Fee Description	Fee Paid
1051	130	Surcharge - late filing fee or oath	
1812	2,520	For filing a request for *ex parte* reexamination	
1251	110	Extension for reply within first month	
1252	420	Extension for reply within second month	
1253	950	Extension for reply within third month	
1254	1,480	Extension for reply within fourth month	
1255	2,010	Extension for reply within fifth month	
1401	330	Notice of Appeal	
1402	330	Filing a brief in support of an appeal	330
1403	290	Request for oral hearing	
1452	110	Petition to revive - unavoidable	
1453	1,330	Petition to revive - unintentional	
1501	1,330	Utility issue fee (or reissue)	
1502	480	Design issue fee	
1460	130	Petitions to the Commissioner	
1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	Submission of Information Disclosure Statement	
8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	Request for Continued Examination (RCE)	
Other fee (specify)			
Other fee (specify)			

SUBTOTAL(3) $330

SUBMITTED BY				Complete (if applicable)	
Typed or Printed Name	Michael D. Yablonsky			Reg. Number	40,407
Signature	[signature]	Date	04/05/2004	Deposit Account User ID	

IN DUPLICATE

Computer generated form "Miscellaneous Fee Sheet" (FEES Folder), Merck & Co., Inc. 10/1/2003

Application Number: 09/469,485

Filing Date: 12/22/1999

First Named Inventor: Qinjian Zhao

Group Art Unit: 1648

Examiner Name: Shanon A. Foley

Attorney Docket Number: 20369Y

FIRST CLASS MAIL CERTIFICATE

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL IN AN ENVELOPE ADDRESSED TO: COMMISSIONER FOR PATENTS, P.O. BOX 1450, ALEXANDRIA, VIRGINIA 22313-1450, ON THE DATE APPEARING BELOW.

MERCK & CO., INC.

MAILED BY Kathy Krajcik DATE 04/05/2004

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Zhao, et al.		
Serial No.:	09/469,485	Case No.:	20369Y
Filed:	December 22, 1999		
For:	IMPROVED RECOMBINANT HEPATITIS B SURFACE ANTIGEN		

Art Unit: 1648

Examiner: Foley, Shanon A.

APPELLANT'S BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Sir:

Appellant appeals the final rejection of claims 8-20. A Notice of Appeal was mailed on February 5th, 2004. This Appeal Brief is submitted in triplicate.

CERTIFICATE OF MAILING

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST CLASS MAIL IN AN ENVELOPE ADDRESSED TO: COMMISSIONER FOR PATENTS, P.O.BOX 1450, ALEXANDRIA, VIRGINIA 22313-1450, ON THE DATE APPEARING BELOW.

MERCK & CO., INC.

BY: Kathy Krajcik DATE: 4/5/04

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application Number: 09/469,485

Filing Date: December 22, 1999

Appellant(s): Zhao, et al.

Examiner: Foley, Shanon A.

APPELLANT'S BRIEF

<u>TABLE OF CONTENTS</u>

LIST OF CITED REFERENCES

Builder et al., US 4,620,948

Even-Chen US 5,242,812

Gavilanes, et al, (1982) JBC, 257, 7770-7777

Petre et al., WO 93/24148 A1

Valenzuela et al., 1979. Nature 280:815-819

Wampler et al. 1985. Proc. Nat. Acad. Sci. 82:6830-6834

REAL PARTY IN INTEREST

The real party in interest is Merck & Co., Inc.

RELATED APPEALS AND INTERFERENCES

There are no related claims and interferences.

STATUS OF CLAIMS

The status of the claims is as follows:

Claims 1-20 are pending in this application.

Claims 1-7 stand cancelled without prejudice to future prosecution.

Claims 8-20 stand rejected based under 35 U.S.C. § 103.

STATUS OF AMENDMENTS

Applicant's Amendment filed on 07 August 2003 has been entered. There are no outstanding Amendments.

SUMMARY OF INVENTION

The present invention provides a method of making an improved recombinant hepatitis B surface antigen, rHBsAg, that has a higher specific antigenicity than previously known rHBsAg. The invention also provides the improved rHBsAg and use of this improved rHBsAg in prophylactic, therapeutic and combination vaccines.

ISSUES

I. Whether Claims 8-16, directed to methods of making an improved recombinant hepatitis B surface antigen, are unpatentable under 35 U.S.C. § 103 over Builder *et al.*, US 4,620,948 in view of Valenzuela *et al.*, 1979. Nature 280:815-819.

II. Whether Claim 17, dependent on Claim 8 and further comprising the steps of adding aluminum adjuvant and co-precipitating; Claim 19, dependent on Claim 8 through Claim 18, limited to a range of incubation times and further comprising the steps of adding formalin and incubating, and the steps of adding aluminum adjuvant and co-precipitating; and Claim 20, dependent on Claim 8 but limited to certain incubation times and further comprising the steps of adding aluminum adjuvant and co-precipitating, are unpatentable under 35 U.S.C. § 103 over Builder *et al.* and Valenzuela *et al.*, as applied to Claims 8-16 in view of Petre *et al.*, WO 93/24148 A1.

III. Whether Claim 18, dependent on Claim 8 but limited to a range of incubation times and further comprising the steps of adding formalin and incubating, is unpatentable under 35 U.S.C. § 103 over Builder *et al.* and Valenzuela *et al.*, as applied to Claims 1-16 in further view of Even-Chen, US 5,242,812.

GROUPING OF CLAIMS

Claims 8-16 recite a method for making rHBsAg starting from cell culture filtrate.

Claims 17, 19 and 20, ultimately dependent on Claim 8 but limited to certain incubation conditions, further comprise the steps adding formalin (Claim 19 only), adding an aluminum adjuvant and co-precipitating the rHBsAg with the aluminum adjuvant.

Claim 18, dependent on Claim 8 but limited to certain incubation conditions and further comprises the steps of adding formalin and incubating.

For purposes of the present Appeal, claims 8-20 stand or fall together.

<u>ARGUMENT</u>

I. <u>Claims 8-16 are patentable under 35 U.S.C. § 103 over Builder *et al.*, US 4,620,948 in view of Valenzuela *et al.*, 1979. Nature 280:815-819.</u>

Claims 8-16 recite a method of making rHBsAg starting from culture filtrate. Applicant summarizes the Examiner's position to be that Valenzuela provides motivation for one of skill in the art to make properly folded HBsAg and that Builder provides a method of refolding proteins into an active conformation.

Both the starting materials and end products of Applicant's method and Builder's method are quite different. Builder started from inactive, improperly folded, insoluble materials and ended up simple monomeric proteins or enzymes. In stark contrast to Builder's teaching, the present method does not start with inactive, insoluble protein or result in monomeric proteins. In the present method, the starting material is soluble, highly complex 22 nm diameter particles composed of about 100 copies of appropriately folded rHBsAg protein plus lipids and carbohydrates. The complexes can be 25-75% lipid (Gavilanes, et al, (1982) JBC, 257, 7770-7777). The complex is very immunogenic and is in wide use today as a vaccine for the prevention of infection by hepatitis B virus. The real party in interest, Merck & Co., markets the starting material in the RECOMBIVAX HB vaccine. In contrast to the method of Builder, in the present method, improved rHBsAg having better defined epitopes is made, without denaturing or disassembling the protein lipid complex particles.

Builder, *et al.*, teaches a method designed, in Builder's own words quoted below, for refolding insoluble proteins which have been produced by host cells as refractile bodies. To wit, Builder *et al.*, states:

"A large number of human, mammalian, and other proteins, including, for example, human growth hormone, (hGH) bovine growth hormone (bGH) and a number of interferons have been produced in host cells by transfecting such cells with DNA encoding these proteins and growing resulting cells under conditions favorable to the expression of the new

heterologous protein. Viral coat proteins, such as capsid proteins of foot and mouth disease (FMD) virus and the surface antigenic protein of hepatitis B virus (HBsAg) are still other examples of heterologous proteins which have also been produced in suitable recombinant DNA engineered hosts. The **heterologous protein is frequently precipitated** inside the cell, and constitutes a significant portion of the total cell protein." (lines 18-32, **emphasis** added).

Further, Builder *et al.*, teach:

"Various heterologous proteins expressed in bacterial host cells, for example, pGH, hGH, and viral coat proteins such as a fusion protein with FMD virus, protein and **HBsAg form refractile bodies** to a greater or lesser extent under commonly found culture conditions. Certain other proteins such as immune interferon (IIF) and leukocyte interferon (LeIF) are more soluble in the cytoplasm. (Fibroblast interferon (FIF) is, however, refractile in host culture.)" (col. 6, lines 48-56, **emphasis** added)

Moreover, Builder *et al.*, states:

"The invention herein is directed to procedures which are useful in isolating, purifying, and, if necessary, reactivating proteins which appear in host cells in the form of "refractile bodies". Part of the invention concerns methods which encourage such refractile body formation; **however, the procedures for protein recovery and activation disclosed herein are intended to be specifically applicable to such refractile proteins**." (col 6, lines 30-37, **emphasis** added)

The rHBsAg of the present invention is present in the above described soluble complex protein lipid particles which are formed by the recombinant yeast host and isolated from the cell filtrate. Builder *et al.*, teaches in both Scheme 1 (col 9-10) and Scheme 2 (Col 18), that in applying the method of Builder's invention, processing the insoluble proteins found in refractile bodies includes steps to centrifuge the cell lysate and discard the supernatant. The starting material of the present invention is soluble, not refractile and would be found in the supernatant which Builder teaches should be discarded.

Applicant cites, in direct and opposite contrast, and the specification points to the method of Wampler *et al.*, 1985. Proc. Nat. Acad. Sci. 82:6830-6834, which requires centrifugation and collection of supernatant. At page 8 and elsewhere, the specification teaches mixing and blending – physical steps carried out on liquids. Example 1, page 19, line 12 refers to "200 mL of SFP in a glass bottle to which a redox buffer is added and "mixed" (line 14). The specification particularly points to the rHBsAg produced by Wampler after the filtration step. Wampler *et al.*, page 6830, right column, heading "Antigen Purification" describes a process of taking a "crude extract", removing debris by centrifugation, and filtering the supernatant. Applicant is unclear as to how the Examiner could read Wampler *et al.*, after having centrifuged and filtered the solution, to have produced anything other than a solution containing soluble complexes of rHBsAg and lipid. Any insoluble protein that may be produced, according to Builder *et al.*, "to a greater or lesser extent," is discarded in Wampler *et al.*, type processes. Therefore, the teaching of Builder *et al.*, is relevant to the refolding of insoluble proteins and "specifically applicable to refractile proteins." The reference is not relevant to the present Wampler type process yielding soluble protein lipid complexes.

In view of the above, Applicant believes that the Examiner has simply not responded to, or perhaps misunderstood, Applicant's remarks of record. Moreover, rather than address the teaching of Wampler *et al.*, a reference believed to be relevant to the present claims as a prior art method of making soluble HBsAg, a reference cited in the specification as the method for making the soluble HBsAg used in the present method and a reference that attempted the use of a redox buffer, the Examiner chooses simply not to cite that reference. Instead, the Examiner cites Valenzuela *et al.* – a reference that provides only motivation to obtain properly folded HBsAg – something already accomplished by Wampler and used as the starting material for the present invention.

Wampler teaches away from the presently claimed method.

Wampler *et al.*, discussed the use of a mixture of oxidized and reduced glutathione to refold proteins. (page 6833, col 2, lines 42-55). Wampler *et al.*, concluded that method did not work to increase disulfide crosslinking in HBsAg and stated that their "results favor an oxidative mechanism for the thiocyanate conversion." (*Id*) Wampler *et al.*, thus explicitly teaches away. In view of the fact that Builder *et al.*, does not teach or use their technique on soluble HBsAg and the fact that Wampler *et al.*, teach that the technique did not improve HBsAg, Applicant contends that one of ordinary skill in the art at the time the invention was made would not have

had a reasonable expectation of being able to produce the instant invention. The reference, like Valenzuela, notes the importance of correct disulfide formation in making recombinant HBsAg. However, unlike Valenzuela *et al.*, Wampler *et al.*, explicitly teaches away from using a redox buffer as presently claimed.

The Examiner stated:

"Wampler et al., do not teach the claimed method of purifying HBsAg. Wampler et al., do not teach the temperature or length of time of incubation required by the claims. How can a completely different method, that did not work, provide motivation or a reasonable expectation of success for the instant method." Final Action at page 2, 3rd paragraph)

Applicant agrees. However, Applicant can not agree that the reference can not be considered in the determination of non-obviousness. A determination of whether claims are obvious or non-obvious is a consideration of many factors. Included in such a consideration is whether the prior art teaches away from the presently claimed invention. Applicant has brought the Wampler reference to the attention of the Examiner and considers the Examiner's refusal to address the teaching of Wampler *et al.*, unresponsive to Applicant's remarks and short circuiting the required analysis under § 103.

The Examiner argues further that Builder et al., teach the same treatment method as the instantly claimed invention. (Final Action, page 3, lines 17-18). Here, the Examiner appears to be wrong on the facts. Applicant points out that the immediately before making that statement, the Examiner correctly described Builder's method as using strong denaturing solutions and completely denaturing proteins in refractile bodies. However, the Examiner incorrectly states that the strong denaturant does not interfere with the biological activity of stability of the proteins. Applicant contends that is impossible. A refractile body is formed of mis-folded, precipitated protein. While such a protein may be very stable in it's precipitated state, Applicant does not see how a protein that is not folded properly can have a biological activity. And that is exactly the point of the method of Builder et al. That method is designed to take precipitated, inactive, improperly folded protein and use strong denaturants to completely denature the protein. That completely denatured protein is then refolded into a biologically active form. In contrast, Applicant starts with active proteins existing in complex protein-lipid particles and uses a redox buffer to shuffle the few remaining mismatched cysteine bonds into their preferred conformations. Applicant's technique is not a denaturation and refolding technique as taught by Builder et al., and therefore is not the "same treatment method" as the Examiner contends.

What Builder et al., does provide is a specific teaching and an expectation that if one of skill in the art is faced with a precipitated, insoluble protein in refractile bodies, one can use a strong denaturant to fully denature the protein and then use 10mM GSH:1 mM GSSH to refold the denatured protein into a form that is biologically active. Builder et al., does not provide an expectation that one of skill in the art could use a redox buffer on a complex protein-lipid particle composed of approximately 100 already biologically active, immunogenic HBsAg proteins to shuffle those proteins into forms that are even more immunogenic. When the prior art on making such biologically active HBsAg containing particles, as taught by Wampler et al., is viewed in conjunction with Builder et al., the art specifically teaches that this type of technique does not work to increase immunogenicity. Therein lies the present invention. Following the methods now claimed, it is now discovered that one can indeed use a redox buffer on particles containing biologically active, immunogenic HBsAg proteins and thereby increase the immunogenicity of said particles. Methods of improving immunogenicity are presently claimed, not methods of denaturing and renaturing proteins as described and claimed by Builder et al.

Valenzuela et al., is seen to provide only motivation to achieve proper folding of recombinant HBsAg. The reference, being from the same field of HBsAg as Wampler et al., is not seen to provide any motivation to go against the teaching of Wampler et al. Therefore, the reference does not make up for the deficiencies of Builder et al., and does not contradict Wampler et al.

Therefore, Builder et al., is not seen to be relevant because that it teaches a method of denaturing and renaturing insoluble proteins, specifically those found in refractile bodies. Because Valenzuela et al., provides only general motivation and the Examiner has mistakenly not considered relevant prior art that specifically teaches away, Applicant believes that the Examiner has not stated a proper case for the *prima facie* obviousness of the present claims.

II. <u>Claims 17, 19 and 20 are patentable under 35 U.S.C. § 103 over Builder *et al.*, US 4,620,948 and Valenzuela *et al.*, 1979. Nature 280:815-819 in view of Petre et al., WO 93/24148.</u>

Claims 17, 19 and 20 were rejected over Builder *et al.* and Valenzuela *et al.*, in view of Petre *et al.*, WO 93/24148 A1. Applicant incorporates herein all of the comments above

as applied to Builder et al., and Valenzuela et al. Because the teaching of adjuvanting cited in Petre et al. does not make up for the deficiency of Builder et al., and Valenzuela et al., as the primary references, the combinations can not provide the basis for a *prima facie* case of obviousness against the patentability of the present claims. Therefore, Applicant respectfully requests that the stated rejection against Claims 17, 19 and 20 be reversed.

III. Claim 18 is patentable under 35 U.S.C. § 103 over Builder et al., US 4,620,948 and Valenzuela et al., 1979. Nature 280:815-819 in view of Even-Chen, US 5,242.812.

Claim 18 was rejected over Builder et al., and Valenzuela et al., in view of Even-Chen, US 5,242,812. Applicant incorporates all of the comments above as applied to Builder et al., and Valenzuela. Because the teaching of the use of formalin cited in Even-Chen does make up for the deficiency of Builder et al., and Valenzuela et al., as the primary references, the combinations can not provide the basis for a *prima facie* case of obviousness against the patentability of the present claims. Therefore, Applicant respectfully requests that the stated rejection against Claim 18 be reversed.

CONCLUSION

Appellant respectfully submits that claims 8-20 are not obvious in view of the cited art. Appellant requests that the Board of Patent Appeals and Interferences reverse the outstanding rejections of claims /under 35 U.S.C. § 103(a).

Please charge deposit account 13-2755 for fees due in connection with this Appeal Brief. If any time extensions are needed for the timely filing of the present Appeal Brief, Appellants petition for such extensions and authorize the charging of deposit account 13-2755 for the appropriate fees.

April 5, 2004

Respectfully submitted,

By ____________

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Appendix A

Claims on Appeal

8. A method of making recombinant hepatitis B surface antigen (rHBsAg) comprising:

a) providing sterile filtered rHBsAg purified from a cell culture,
b) adding a redox buffer to the rHBsAg,
c) adjusting the temperature to 34 to 38 C,
d) incubating the rHBsAg at 34 to 38 C for 40 to 240 hours.

9. The method of Claim 8 wherein step c is performed before step b.

10. The method according to Claim 8 wherein the redox buffer comprises thiol compounds selected from the group consisting of thiol compounds having a MW less than about 1000 Da and the corresponding disulfide compounds.

11. The method according to Claim 10 wherein the redox buffer is a mixture of at least one thiol compound and at least one disulfide compound.

12. The method according to Claim 11, wherein the ratio of thiol compound to disulfide compound is between about 30:1 and about 1:1.

13. The method according to Claim 12 wherein the concentration of thiol compound is between about 0.05 mM and about 5.00 mM.

14. The method according to Claim 13 wherein the ratio of glutathione to oxidized glutathione is selected from the group consisting of about 20:1, about 10:1, about 10:4, about 5:1, about 2:1 and about 1:1.

15. The method according to Claim 13 wherein the thiol compound is glutathione and the disulfide compound is oxidized glutathione.

16. The method according to Claim 15 wherein the concentration of glutathione is about 1.0 mM and the concentration of oxidized glutathione is about 0.2 mM.

17. The method according to Claim 8 further comprising the steps of
e) adding an aluminum adjuvant, and
f) co-precipitating the rHBsAg and the adjuvant.

18. The method according to Claim 8 further comprising the steps of
e) adding about 0.01% final concentration of formalin ,
f) incubating the rHBsAg at about 34 C to about 38 C from about 40 to about 72 hours,

wherein the incubation in step d is from about 40 to about 190 hours.

19. The method according to Claim 17 further comprising the steps of
g) adding an aluminum adjuvant, and
h) co-precipitating the rHBsAg and the adjuvant.

20. The method according to Claim 8 wherein the incubation in step d is about 60 hours and further comprising the steps of
e) adding an aluminum adjuvant, and
f) co-precipitating the rHBsAg and the adjuvant,
wherein step f includes an incubation of about 40 hours.

Appendix B

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 13, Issue of July 10, pp. 7770-7777, 1982
Printed in U.S.A.

Structure of Hepatitis B Surface Antigen

CHARACTERIZATION OF THE LIPID COMPONENTS AND THEIR ASSOCIATION WITH THE VIRAL PROTEINS*

(Received for publication, December 8, 1981)

Francisco Gavilanes, Jose M. Gonzalez-Ros‡, and Darrell L. Peterson§

From the Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

The lipid composition of hepatitis B surface antigen (HBsAg) (subtype adw) obtained from different carriers has been determined and proven to be truly characteristic of HBsAg and not subject to individual variation. Phosphatidylcholine (~60%), cholesteryl ester (~14%), cholesterol (~15%), and triglycerides (~3%) are the main HBsAg lipid constituents. The fatty acid composition of the different HBsAg lipid components is similar to that of other normal human serum lipoprotein.

A photoactivatable hydrophobic probe, pyrenesulfonyl azide, has been used to determine what portions of the protein components of HBsAg are exposed to the HBsAg lipid matrix. Both major HBsAg protein components became randomly pyrenesulfonyl azide labeled in both the NH_2-terminal and COOH-terminal tryptic fragments, therefore suggesting they are buried within the HBsAg lipids. A model for the arrangement of proteins in HBsAg is proposed whereby regions within the NH_2-terminal and COOH-terminal parts of the two major HBsAg protein components are buried within the lipid matrix of HBsAg particles, while the antigenically important residue 122–150 region is exposed to the aqueous environment.

Hepatitis B surface antigen is a group of morphologically heterogeneous complex macromolecular structures found in the serum of patients with hepatitis B virus infection. Electron microscopy reveals three forms of HBsAg,[1] 20-nm spherical particles, tubular structures of variable lengths and a diameter of 20 nm, and 40-nm spherical "Dane" particles which are the hepatitis B virus (1-5). Of these, the 20-nm spherical particles account for the bulk of the HBsAg and are the material obtained by our purification procedures. Purified HBsAg has a molecular weight of 2-4 × 10^6 and a density of 1.20-1.21 g/ml (6, 7). Previous reports have shown HBsAg to contain protein, carbohydrate, and lipid (8, 9). Of these components, only the proteins have been well characterized (10). Indeed, a detailed description of the overall molecular composition of HBsAg has not been presented.

Much of the interest in HBsAg arises from its anticipated role in the development of a vaccine against hepatitis B virus. Currently such experimental vaccines utilize: (i) HBsAg, treated to inactivate residual hepatitis B virus (11); (ii) isolated HBsAg p-25 or gp-30 proteins (12); or (iii) synthetic peptides based on the amino acid sequence of HBsAg p-25 protein (13, 14). However, some concern has been voiced concerning the use of isolated whole HBsAg because of the possibility (although perhaps remote) of adverse autoimmune or alloimmune reactions due to the possible presence of host proteins in the purified HBsAg (actually, this vaccine has recently been approved by United States Food & Drug Administration). On the other hand, isolated HBsAg proteins, p-25 and gp-30, are poorly immunogenic (12). Nevertheless, the immunogenicity of these proteins is markedly improved when they are incorporated into lipid micelles (15). It might be expected that synthetic peptides would also be more immunogenic if presented in the form of lipid-peptide particles. Despite the apparent role of lipids in potentiating the immunogenicity and modifying the antigenicity (16) of HBsAg, no detailed information concerning the lipid composition of HBsAg has been reported. Indeed, the only information available in the literature refer to the mere identification of the major lipid classes present (8, 17). Also, no information concerning the interrelation between the protein and lipid components of HBsAg has been presented. For these reasons, we have undertaken a detailed study of the lipids of HBsAg. In addition to identifying the lipid components of HBsAg, we have used a photoactivatable probe, pyrenesulfonyl azide, to obtain data on the interaction of these lipids with the protein components of HBsAg. Pyrenesulfonyl azide partitions almost exclusively into hydrophobic lipid regions and generates a highly reactive nitrene upon exposure to UV light which covalently binds to adjacent constituents. This lipophilic probe has proven useful for studying the localization of membrane-associated proteins in vesicular stomatitis virus (18) and as a marker for the acetylcholine receptor subunits which are in contact with the membrane hydrophobic environment (19, 20). In this paper, we demonstrate that this probe is also useful for obtaining data concerning the interaction of the major protein (p-25) and glycoprotein (gp-30) components of HBsAg with its lipid matrix.

EXPERIMENTAL PROCEDURES

Materials

All electrophoresis reagents were obtained from Bio-Rad. Trypsin was obtained from Sigma. Pyrenesulfonyl azide was purchased from Molecular Probes.

Methods

Assay of HBsAg—HBsAg was assayed by counterelectrophoresis against anti-HBsAg antibodies prepared from immunized guinea pigs.

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‡ Recipient of Research Grant NS 17029 from the National Institutes of Health in support of this work.

§ Recipient of Research Grant AI 15955 from the National Institutes of Health in support of this work.

[1] The abbreviations used are: HBsAg, hepatitis B surface antigen; PySA, pyrenesulfonyl azide; SDS, sodium dodecyl sulfate; HDL, human high density lipoprotein; TLC, thin layer chromatography; GLC, gas-liquid chromatography; PC, phosphatidylcholine.

Isolation of HBsAg—HBsAg used in these studies was obtained from the plasma of different carriers of HBsAg (adw subtype) following previously described procedures (10). The purified HBsAg samples were extensively purged with argon and stored at 3–4 °C. Storage of samples under argon proved to be important in studies involving fatty acid determination. In fact, an overall decrease in the average number of double bonds while maintaining identical chain lengths was observed in samples stored without such a precaution. This was especially noticeable for linoleic acid, the major unsaturated acyl residue of HBsAg.

Lipid Extraction, Fractionation, and Characterization—The total lipid from HBsAg preparations was extracted by the procedure of Bligh and Dyer (21). Lipid extracts were washed with a 0.73% NaCl solution and evaporated to complete dryness. The total lipid content of HBsAg was determined gravimetrically. Dried lipid extracts were taken up to approximately 25 mg of lipid/ml in choloroform:methanol (2:1, v/v) and stored under argon at −30 °C until used for further analysis (between 1–7 days). Handling of lipid extracts was always done under argon, and all organic solvents were of high quality and glass-distilled from Burdick and Jackson Laboratories, Inc. Total, free, and esterified cholesterol and phosphorus content were determined simultaneously in the lipid extracts to avoid errors from evaporation of the chloroform:methanol solutions. Cholesterol determinations were done by a more sensitive modification of the Zlatkis *et al.* (22) and Courchaine *et al.* (23) procedures. Total lipid aliquots (6–12 μl from stock solutions) or chloroform eluates from cholesterol and cholesteryl ester TLC spots were deposited in conical centrifuge tubes, dried under a stream of argon, and dissolved in 3 ml of glacial acetic acid. Two ml of a dilute $FeCl_3$ color reagent (21 ml of concentrated H_2SO_4 plus 4 ml of a solution of 500 mg of $FeCl_3 \cdot 6H_2O$/20 ml of 85% phosphoric acid) were added and mixed, and the absorbances at 550 nm were determined after 30 min at room temperature. The calibration curve was obtained with free cholesterol (Sigma) dissolved in acetic acid (10–200 μg of cholesterol). Free cholesterol was also determined by the same procedure making use of the specific formation of a digitonide precipitate from an acetone solution of the free sterol in the presence of digitonin (Sigma). Levels of cholesteryl esters relative to the other fatty acid-containing lipids were also determined by GLC in the presence of an internal standard.

Lipid phosphorus was determined by the micromethod described by Bartlett (24) except that the Fiske and SubbaRow reagent was prepared by dissolving 6.25 mg of 1-amino-2-naphthol-4-sulfonic acid and 5 g of $Na_2S_2O_5$ up to 25 ml with distilled water. Absorbances at 830 nm were measured and interpolated into a calibration curve obtained from a KH_2PO_4 solution. This method allows for accurate quantitation of as little as 50 ng of phosphorus. Silica gel contained in phospholipid spots from TLC plates was eliminated prior to the determination of the absorbances at 830 nm by extensive centrifugation in a clinical tabletop centrifuge. Under these conditions, presence of silica gel did not interfere with the colorimetric procedure.

Neutral lipid classes were fractionated by TLC on activated (1 h, 110 °C) 0.25-mm layers of Silica Gel G (Redi-Plate, Analtech) using *n*-hexane/ethyl ether/acetic acid/methanol (60:40:1:1, by volume) as the developing solvent. After development, the plates were sprayed with a 0.05% rhodamine solution in methanol, and lipid bands were visualized under UV light. In all cases, chromatography chambers were lined with filter paper saturated with solvent for at least 1 h prior to use. Migration distances were compared with those of commerical standards (Sigma or Supelco) on the same or parallel plates.

Polar lipids were fractionated by two-dimensional TLC on activated 0.2-mm layers of linear high performance plates (10 × 10 cm; LHP-K, Whatman). Aliquots containing 100–150 μg of total lipid were developed twice (developed, dried under a stream of argon, then developed again) using chloroform/methanol/concentrated ammonia (65:25:4, by volume). Plates developed twice in the first dimension were dried for 15 min under a stream of argon and 30 min under vacuum, and then developed with chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5, by volume) in the second dimension. It was crucial that all traces of NH_4OH be removed prior to chromatography in the second dimension. Lipid spots were visualized by charring with 10 N H_2SO_4 (for samples for phosphorus determinations or by spraying with Phospray (Supelco) (for identification of phosphorus-containing lipids) or with a 0.05% methanol:water (1:1, v/v) solution of 1′,7′-dichlorofluorescein (for samples for fatty acid analysis). Quantitation of the different diacyl phospholipids *versus* the corresponding alk-1-enyl (plasmalogens) derivatives was achieved through a separation-reaction-separation two-dimensional TLC procedure similar to the one described by Owens (25). This method is based on the labile character of plasmalogens to hydrolysis by acids or by $HgCl_2$ while the diacyl phospholipids are stable. The lipids are first applied on LHP-K Whatman plates and chromatographed in the first dimension as described above, and then the portion of the plate containing the fractionated lipids is sprayed with a 0.05 M solution of $HgCl_2$ and kept under a stream of argon for 30 min at room temperature (reaction step). The plates are then dried and developed in the second dimension as described above, which resulted in the concomitant separation of each phospholipid class (separated in the first dimension) and also of acid-stable (diacyl phospholipids remaining with the same R_F) and acid-labile subpopulations (as lysoforms) for each phospholipid class. Phosphorus determinations on the lipid spots allow for quantitation of the ratio of diacyl phospholipid to plasmalogen.

For preparative purposes, PC was isolated from lipid extracts by TLC on 0.25-mm layers of Silica Gel G (Redi-Plate, Analtech) using chloroform/methanol/concentrated ammonia (65:25:4, by volume) as the solvent system. The PC band was scraped off the plate and exhaustively eluted from the adsorbent as previously described (26). The positional distribution of the acyl groups in the isolated PC was determined by hydrolysis with phospholipase A_2 (EC 3.1.1.4) present in *Crotalus adamanteus* venom (Sigma) as basically described by Van Golde and Van Deenen (27). Fatty acid analysis of the initial PC and its lyso derivative (sn-1 position) was used for the calculations.

Transmethylation of total or fractionated lipid classes was performed with a sodium methoxide/methanol reagent (methanolic base, Supelco). The reagent (1 ml) was added to the dried lipid samples and kept under argon for 15–18 h at room temperature in the dark. Traces of silica gel, rhodamine, or dichlorofluorescein did not affect the transesterification or the resulting products. Fatty acid methyl esters were extracted in a water-petroleum ether system, taken to dryness under argon in small conical centrifuge tubes, and finally dissolved in 20-μl aliquots of isooctane for analysis by GLC.

GLC analysis of fatty acid methyl esters was performed on a Varian 3700 gas chromatograph equipped with a dual flame ionization detector and with 6-foot glass columns (2 mm internal diameter). Quantitation of the peak areas was achieved by automatic integration with a Hewlett-Packard 3390-A recording integrator. 10% SP-2340 cyanosilicone on 100–120 Supelcoport (Supelco) was used over a temperature range of 142–234 °C (4 min at 142 °C, linear increase from 142–234 °C at 4 °C/min, and 8 min at 234 °C) and a carrier gas flow (N_2) of 50 ml/min. Components in the chromatographed samples were identified by comparison of the retention time with known standards (Supelco and Sigma). Methyl pentadecanoate was used as an internal standard.

PySA Labeling Procedure—HBsAg at a concentration of 1.5 mg of protein/ml in 10 mM sodium phosphate buffer, pH 7.4, 0.1 mM EDTA, and 0.02% NaN_3 was placed in a 3-ml thick walled quartz cuvette containing enough dried PySA to provide a final concentration of 2 mM. All operations with PySA were performed in the dark. The mixture was stirred at room temperature until no further increase in the absorbance at 365 nm was observed (approximately 75 min). Excess PySA remaining insoluble was then removed by low speed centrifugation in a clinical centrifuge. The suspension was then exposed for 45 min to UV light (>300 nm) from a Mineral Light UVS 58 (Ultraviolet Products, Inc.) with the filter removed. A gentle stirring of the sample was maintained during the irradiation to avoid inner filter effects. The distance between the sample cuvette and the light source was approximately 3 cm. Alternatively, [^{3}H]PySA (18 Ci/mol specific activity) prepared as described elsewhere (19) was used to quantitate the extent of incorporation of PySA into HBsAg particles or its protein components. [^{3}H]Toluene was used as an internal standard in determining the efficiency of the counting. Upon irradiation, the labeled HBsAg particles were separated from nonincorporated PySA photoproducts (solubility in aqueous solutions, 10^{-5}–10^{-6} M) by passage through a Sepharose 2B column (1.5 × 45 cm) and equilibrated in the above-mentioned phosphate buffer (Fig. 3). Approximately one-third of the available PySA was found incorporated into HBsAg particles. Irradiation was also performed in the presence of a 2-fold excess (w/w) of bovine serum albumin over HBsAg protein to prevent possible PySA labeling of the HBsAg components coming from the aqueous medium rather than from within the HBsAg particle. The distribution of the [^{3}H]PySA label on the protein components of HBsAg was measured after SDS-polyacrylamide slab gel electrophoresis where either a 12–23% linear gradient or a 5–22% exponential gradient polyacrylamide gel was used. A 4% polyacrylamide stacking gel was used in either case. The gel and buffer formulations were those of O'Farrell (28). Upon visualization of the

electrophoresed gel under UV light, the gel was cut into strips and placed into scintillation vials containing 1 ml of 2% SDS. After overnight incubation and shaking of the samples at 37 °C, scintillation fluid was added, and the samples were counted.

Preparative polyacrylamide gel electrophoresis was performed using 12-22% polyacrylamide linear gradient slab gels. Upon location of the proteins under UV light, the protein strips were cut and placed in an electrophoretic apparatus as described by Allington *et al.* (29) where they were electrophoretically eluted from the gel and concentrated into a 0.15 M ammonium hydroxide solution containing 0.1% SDS. Generally, 1 h at 140 V was sufficient for a complete elution of the protein from the gel strip. SDS was then removed by the addition of 20 volumes of acetone:triethylamine:acetic acid (85:5:5, by volume) as described by Henderson *et al.* (30). Tryptic digestion of the isolated p-25 and gp-30 was performed according to previously published procedures (10). The digestion was allowed to proceed for 12 h with constant stirring at 37 °C. Following digestion, the hydrolysis mixtures were taken to dryness in a Savant Speed-Vac concentrator, and the hydrolysis products were examined by SDS-polyacrylamide gel electrophoresis.

Absorption and fluorescence emission spectra were taken in a Cary 210 (Varian) spectrophotometer and in a SLM 4000 spectrofluorimeter, respectively.

RESULTS

Purification of HBsAg—Electron micrographs obtained from HBsAg preparations purified as described under "Methods" showed a homogeneous population of particles 20 nm in diameter (Fig. 1*A*). Purified HBsAg exhibited a major protein, p-25 (M_r = 25,000), and a major glycoprotein, gp-30 (M_r = 30,000) (Fig. 1*B*), which accounted for more than 90% of the total protein content of the antigen. Amino acid sequencing data from both p-25 and gp-30 components recently demonstrated that gp-30 is the glycosylated derivative of p-25; hence, both contain the same polypeptide chain (10). Assuming identical molar extinction coefficients for both p-25 and gp-30, we have estimated an apparent p-25:gp-30 stoichiometry of approximately 2:1 by scanning at 280 nm of unstained 12-23% linear gradient polyacrylamide gels following electrophoresis of the purified antigen (Fig. 1*B*).

Lipid Composition of HBsAg—For the studies reported here, plasma from different patients known to be HBsAg carriers was utilized to obtain several HBsAg preparations. Samples 1, 3, and 5 were obtained from three different individuals. Sample 6 originated from a pool of equal amounts of

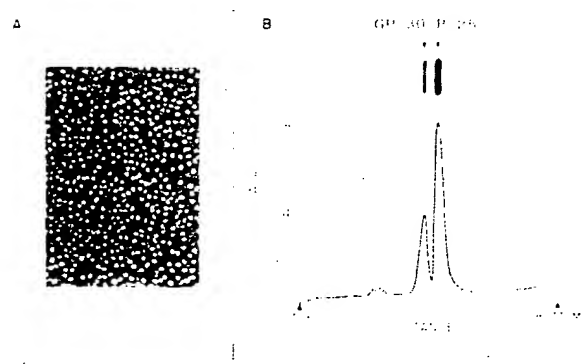

FIG. 1. **Purified HBsAg.** *A*, negative stain electron micrograph of an HBsAg sample purified as described under "Methods." A 1% solution of sodium phosphotungstate was used as the negative staining agent. *B*, SDS-polyacrylamide gel electrophoresis of purified HBsAg. The sample was dissolved in 25 μl of 62.5 mM Tris-HCl, pH 6.8, containing 10% mercaptoethanol and 2.3% SDS, and then heated in boiling water for 1 min. The solution was then made 10% in glycerol containing bromphenol blue as tracking dye and electrophoresed on a 0.75-mm thick 12-23% linear gradient polyacrylamide gel at 15 mA constant current until the dye eluted. The *lower part* of *B* shows a 280-nm scan of an unstained gel run under the conditions from above.

HBsAg obtained from the plasma of two different carriers. Samples 2 and 4 correspond to individuals 1 and 3, respectively, except that plasma was withdrawn several weeks later. Table I shows the lipid to protein and phospholipid to cholesterol ratio for the six different preparations of HBsAg as well as the averages and standard deviations calculated from all of them. All HBsAg samples analyzed exhibited lipid to protein and phospholipid to cholesterol ratios whose individual values do not significantly differ from each other, thus having standard deviations which ranged well within experimental error. A high degree of analogy was also observed in the different samples with regard to the total fatty acid composition of lipids extracted from HBsAg preparations (Table II). The major fatty acid components were palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids which accounted for more than 85% of the total fatty residues.

On the basis of these determinations, it can be concluded that lipids associated to HBsAg particles exhibit a defined compositional pattern and are not subject to individual variations. Consequently, the lipid data in subsequent tables (III and IV) are given as the averages of several separate determinations rather than on an individual basis.

The major lipid classes present within HBsAg particles (Table III) are phospholipids (~67%), free (~15%) and esterified (~14%) cholesterol, and triglycerides (~3%). The molar abundances of phospholipids and cholesteryl esters were determined by both colorimetric procedures and by GLC in the presence of an added internal standard (methyl pentadecanoate). Both methods provided values which agreed very closely. Free cholesterol was determined following two colorimetric approaches, while triglycerides relative levels were determined by GLC in the presence of an internal standard (see "Methods").

The fatty acid composition of each major lipid class is also shown in Table III. As expected from its high relative abundance, the compositional pattern observed in the fatty acids

TABLE I
Total Lipid Contents of HBsAg

	Preparation						Average ± S.D.
	1	2	3	4	5	6	
Lipid/protein[a] (weight ratio)	0.41	0.28	0.39	0.31	0.32	0.35	0.34 ± 0.05
Phospholipid/cholesterol[b] (molar ratio)	2.10	2.11	2.00	2.25	1.86	2.31	2.10 ± 0.16

[a] Total lipid and proteins were estimated gravimetrically and using the value of 3.72 as the absorbance at 280 nm for a 1 mg/ml solution of HBsAg (37), respectively.

[b] Total phosphorus and total cholesterol were determined by colorimetric procedures (see "Methods").

TABLE II
Fatty acid composition of total lipids from HBsAg (weight %)

	Preparation						Average ± S.D.
	1	2	3	4	5	6	
16:0	29.5	24.2	29.6	24.7	28.5	25.4	27.0 ± 2.3
16:1	2.0	3.2	1.9	3.5	3.5	1.6	2.6 ± 0.8
18:0	13.0	11.3	12.3	12.6	12.4	15.1	12.8 ± 1.2
18:1	17.7	16.4	20.9	23.2	25.2	19.7	20.5 ± 3.0
18:2	30.7	35.0	26.6	23.4	24.3	27.1	27.9 ± 3.9
22:1	2.5	3.0	1.9	3.6	1.6	2.7	2.6 ± 0.7
20:4	3.9	4.8	6.0	7.6	4.0	7.7	5.7 ± 1.6
22:6	0.8	2.1	0.9	1.5	0.5	0.8	1.1 ± 0.5
Average degree of unsaturation[a]	1.0	1.2	1.1	1.2	1.0	1.1	1.1 ± 0.1
Average chain length[a]	17.5	17.8	17.6	17.8	17.5	17.7	17.7 ± 0.1

[a] Average degree of unsaturation and chain length in Tables II-IV refer to the fatty acyl chains.

TABLE III

Fatty acid composition of major lipid classes (weight %)

The values indicated are the averages and standard deviations for preparations 1, 2, 3, and 5. TG, triglycerides; PL, phospholipids; CHE, cholesterol esters; CH, cholesterol.

	TG	PL	CHE	CH	Total recovery of fatty acid[a]
16:0	26.8 ± 3.8	28.5 ± 1.5	12.0 ± 1.5		25.7
16:1	6.8 ± 1.4	0.9 ± 0.2	4.7 ± 2.5		1.8
18:0	3.5 ± 1.3	15.9 ± 1.7	1.3 ± 0.4		12.9
18:1	41.0 ± 3.9	15.7 ± 2.2	22.0 ± 2.7		17.8
18:2	21.9 ± 1.3	28.0 ± 4.5	52.8 ± 3.4		31.9
22:1	ND[b]	2.3 ± 0.4	3.7 ± 1.5		2.4
20:4	ND	7.0 ± 1.7	3.5 ± 1.4		6.1
22:6	ND	1.7 ± 0.6	ND		1.3
Molar percentage[c]	3.4 ± 1.0	66.9 ± 1.8	14.2 ± 1.1	15.5 ± 1.2	
Average degree of unsaturation	0.9 ± 0.0	1.1 ± 0.1	1.5 ± 0.1		1.1
Average chain length	17.3 ± 0.1	17.7 ± 0.1	17.8 ± 0.1		17.8

[a] Recovery of fatty acids is calculated on the basis of the molar relative abundances and fatty acid composition of TG, PL, and CHE by a_i total $= \sum a_{ij} \times P_j/100$, where "$a_i$ total" represents the recovery of the different "i" fatty acids in the total lipid fraction. "a_{ij}" are the weight percentages of each "i" fatty acid in each "j" lipid class and "P_j" are the molar percentages of each "j" fatty acid containing lipid class. The values of 4.0, 79.2, and 16.8% were used as the molar percentage for TG, PL, and CHE, respectively and were obtained from the percentages given above excluding the contribution of free cholesterol.

[b] ND, not detected.

[c] Molar percentage of each lipid class based on single determination by GLC using an internal standard and colorimetric procedures (see "Methods").

present in the phospholipid fraction resembles that observed for total HBsAg lipids. Palmitic and linoleic acids (~28% each) and stearic and oleic acids (~15% each) were the major fatty residues in phospholipids, providing an average chain length of 17.7 carbons and approximately 2 double bonds per phospholipid molecule. Cholesteryl esters and triglycerides contain linoleic (~52%) and oleic (~41%) acids, respectively, as the major acyl components.

From the fatty acid composition of the phospholipids, cholesteryl esters, and triglycerides, and from the relative abundance of these components, the recovery of acyl residues throughout the analytical process can be calculated. As shown also in Table III, such a recovery accounts for most acyl residues of the experimental total fatty acid composition shown in Table II, therefore validating the techniques used for the analysis and indicating that most HBsAg lipid components were accounted for.

The phospholipid types present in the HBsAg phospholipids fraction are summarized in Table IV. The recovery of phosphate from the TLC plates was within the 90–100% range; therefore, the possibility of the existence of undetected phospholipids in the lipid extracts is unlikely. Choline phosphoglycerides accounted for approximately 90% of the total phospholipids and were almost exclusively composed of $HgCl_2$-stable diacyl derivatives (PC) since only 6% of the choline phosphoglyceride population became hydrolyzed to a lyso derivative upon treatment with $HgCl_2$ (presumably, choline plasmalogens). Conversely, ethanolamine phosphoglycerides accounted for only 2–4% of the total phospholipids and were composed of approximately equal amounts of $HgCl_2$-stable (phosphatidylethanolamine) and $HgCl_2$-labile (ethanolamine plasmalogens) forms. Minor phospholipid components were phosphatidylserine, sphingomyelin, lysophosphatidylcholine, and lysophosphatidylethanolamine. Phosphatidylinositol or cardiolipin were not detected in any preparation. Table IV also summarizes the fatty acid composition of the individual phospholipid classes. As expected from its high relative abundance, PC exhibits a fatty acid composition very similar to the one previously shown in Table III for the total population of phospholipids. Other phospholipids such as phosphatidylethanolamine or phosphatidylserine contain slightly longer fatty chains on the average than PC, although the average

TABLE IV

Fatty acid composition of phospholipid classes (weight %)

The values indicated are the average and standard deviations for preparations 1, 2, 3, and 5. PE, phosphatidylethanolamine; PS, phosphatidylserine; SPH, sphingomyelin; 1-PC, lysophosphatidylcholine; 1-PE, lysophosphatidylethanolamine.

	PC	PE	PS	SPH + 1-PC + 1-PE
16:0	31.6 ± 3.1	22.9 ± 0.2	16.2 ± 0.7	25.1 ± 0.9
16:1	1.1 ± 0.5	12.3 ± 2.1	3.5 ± 2.0	10.3 ± 1.4
18:0	15.7 ± 2.3	15.8 ± 0.4	26.6 ± 2.2	14.7 ± 1.1
18:1	17.4 ± 2.3	21.1 ± 2.2	17.0 ± 1.5	16.2 ± 0.7
18:2	25.8 ± 3.0	12.2 ± 0.5	8.8 ± 0.4	7.6 ± 1.2
22:1	2.4 ± 0.5	7.0 ± 0.6	16.3 ± 1.0	26.1 ± 4.9
20:4	4.4 ± 1.6	8.8 ± 2.7	11.7 ± 2.6	TR[a]
22:6	1.6 ± 0.3	ND[b]	ND	ND
Average degree of unsaturation	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.7 ± 0.1
Average chain length	17.6 ± 0.2	17.8 ± 0.1	18.5 ± 0.2	18.3 ± 0.3
Molar percentages[c]	87.8 ± 0.3	1.7 ± 0.8	TR	10.5 ± 1.3
Molar percentages[d]	90.8 ± 2.0	4.0 ± 1.2	1.8 ± 1.1	3.3 ± 1.8

[a] TR, traces, molar abundances lower than 0.5%.

[b] ND, not detected.

[c] Phospholipid composition based on duplicate determinations of phosphorus contents by Bartlett's procedure.

[d] Phospholipid composition based on single determinations by GLC using an internal standard.

degree of unsaturation remained fairly constant at approximately two double bonds per phospholipid molecule.

Since PC, in addition to free and esterified cholesterol, is a major lipid component of HBsAg particles, we have investigated further its chemical structure by making use of the selective cleavage of *sn*-2 positioned fatty chains by phospholipase A_2 from snake venoms. The positional distribution of acyl chains between the *sn*-1 and *sn*-2 positions of PC is depicted in Fig. 2. Main features regarding the major fatty acid components are the selective positioning of linoleic and stearic acids at *sn*-2 and *sn*-1 positions, respectively. Conversely, palmitic acid is preferentially located at the *sn*-1 position, while oleic acid is equally distributed between both *sn*-1 and *sn*-2 positions.

Studies with Pyrenesulfonyl Azide—When PySA and/or [^{3}H]PySA was incorporated into HBsAg particles and then

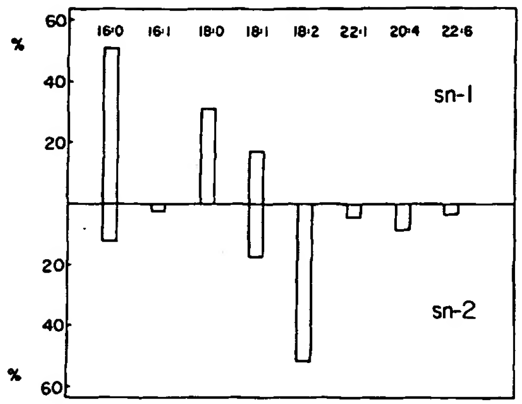

FIG. 2. **Positional distribution of acyl groups in PC from HBsAg lipids.** The *upper panel* indicates the fatty acid composition at the *sn*-1 position of PC as obtained from fatty acid analysis of the lyso-PC derivative resulting from phospholipase A_2 hydrolysis of the original PC. The *lower panel* indicates the fatty acid composition at the *sn*-2 position of PC as calculated from the difference between the fatty acid composition of the intact PC (Table IV) and that at the *sn*-1 position.

irradiated for 45 min under the conditions described under "Methods," about 80% of the photoactivatable probe became photolyzed to reactive nitrenes. Since complete photolysis of PySA would take approximately 45 min more, we decided to use the above conditions of 80% photolysis to avoid possible damage of the protein components of HBsAg by prolonged ultraviolet exposure. Under these conditions, the PySA-labeled antigen preparation completely retained its antigenic activity as judged by counterelectrophoresis against anti-HBsAg. After removal of the nonsolubilized hydrophobic probe from the irradiated mixture, the soluble nonincorporated [^{3}H]PySA photolysis products (the solubility limit of PySA or PySA photoproducts in aqueous solutions is in the 10^{-5}–10^{-6} M range) were separated from [^{3}H]PySA-labeled HBsAg particles by chromatography on Sepharose 2B (Fig. 3). The first peak centered around fraction number 37 and contained approximately one-third of the total radioactivity applied to the column. The absorbance spectrum of a pool from fractions 25 to 47 is shown in Fig. 4; the 280-nm region of the spectrum coincides almost exactly with that of purified HBsAg alone. The specific activity of the [^{3}H]PySA-labeled HBsAg was approximately 0.085 μmol of PySA/mg of protein (about 10^6 cpm/mg of protein). On the other hand, the absorption spectrum of pooled fractions 48–62 (Fig. 4) was identical with the one observed for the PySA photoproducts obtained by irradiation in the absence of HBsAg particles. When the irradiation was performed in the presence of water-soluble scavengers (bovine serum albumin, 2-fold excess by weight over HBsAg protein), the extent of PySA incorporation into HBsAg remained unchanged. Furthermore, the ratio of absorbances at 280 nm to 364 nm (as indicative of protein/PySA ratio) upon irradiation in the presence of the albumin was identical with that obtained in its absence.

Polyacrylamide gel electrophoresis in the presence of SDS and reducing agents such as mercaptoethanol is the only method by which we have been able to separate and, subsequently, isolate p-25 and gp-30 (31). The distribution of [^{3}H] PySA photoproducts among HBsAg components was determined after SDS-gel electrophoresis. The electrophoretic pattern of HBsAg protein components (protein stain) is not changed by photolabeling, which indicates that no apparent loss of polypeptide material occurred as a result of cross-linking or any other kind of polymerization or hydrolysis reaction. Both major protein components, p-25 and gp-30, were labeled (Fig. 5) as demonstrated by the incorporation of radioactivity and by the fluorescence of both p-25 and gp-30 bands. Radioactivity measurements and scanning at 354 nm of unstained gels gave a ratio of p-25 to gp-30 bands of approximately 2:1 which coincides with the p-25/gp-30 stoichiometry obtained by 280-nm absorbance scans (Fig. 1*B*). These results indicated that both protein and glycoprotein were labeled by PySA to the same extent and suggested their buried character within the lipid matrix. The specific activities of both protein components were approximately 0.011 μmol of PySA/mg of protein. Nevertheless, most of the radioactivity incorporated into HBsAg particles is associated with nonprotein components. About 87% of the incorporated radioactivity was found to be associated with a fluorescent band that did not stain with Coomassie blue and migrated close to the dye front in SDS gels. This is likely to be associated with the lipid components of HBsAg as previously demonstrated with the acetylcholine receptor system (19).

PySA-labeled p-25 and gp-30 were electrophoresed out of the gels into a buffered solution containing SDS. The fluorescence emission spectra of both purified labeled components is

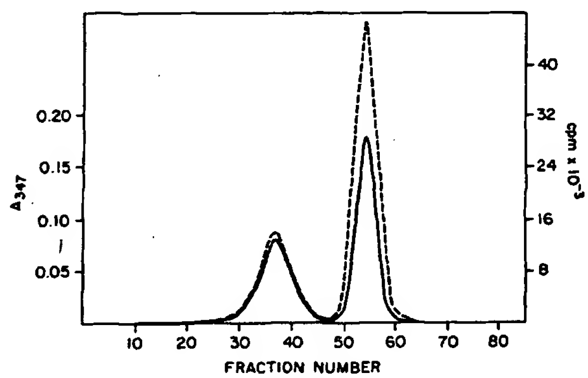

FIG. 3. **Removal of nonassociated [^{3}H]PySA photoproducts from [^{3}H]PySA-labeled HBsAg particles after irradiation.** Gel filtration chromatography in Sepharose 2B column (see "Methods") was performed. Fractions of 1.2 ml were collected. ——, represents the continuous monitoring of absorbance at 280 nm; - - -, indicates the radioactivity of 100-μl aliquots of each fraction.

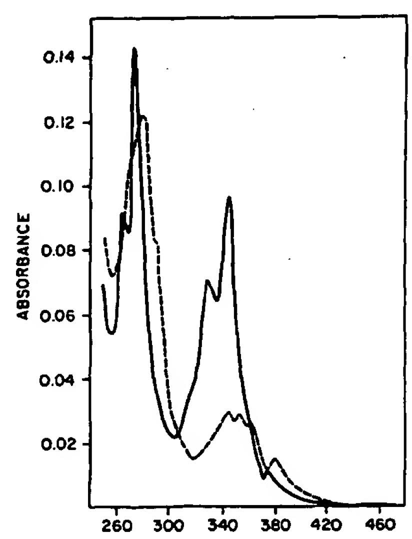

FIG. 4. **Absorption spectra of pooled fractions from Sepharose 2B column.** ——, represents the spectrum of pooled fractions 48–62; - - -, represents the spectra of pooled fractions 25–47.

shown in Fig. 6. Upon excitation at the excitation maximum (346 nm), labeled protein and glycoprotein exhibited emission maxima at 385, 404, and 424 nm which are in agreement with the results obtained by Sator *et al.* (19). When compared to the emission spectrum of the intact labeled HBsAg particle, these emission maxima were shifted toward longer wavelengths indicating a more polar environment of the fluorophore in the presence of SDS than in the native HBsAg particle. Also, an increased 385 to 404 nm ratio, characteristic of protein-associated PySA, was observed in the SDS-purified samples.

Tryptic hydrolysis of either purified protein component of HBsAg, p-25 and gp-30, does not give rise to soluble peptides even after prolonged digestion (about 18 h) at 37 °C with

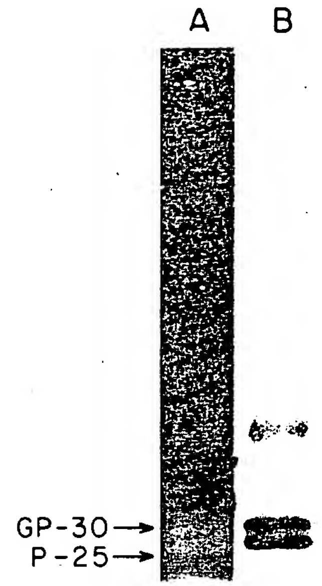

FIG. 5. **SDS-polyacrylamide gel electrophoresis of PySA-labeled HBsAg.** The samples were treated, and the electrophoresis was performed as described for Fig. 1, except that a 5–23% exponential gradient polyacrylamide gel was used. *Lane A* shows the unstained gel visualized under long wavelength UV light. *Lane B* shows the same gel as lane A, except that it is now stained with Coomassie blue.

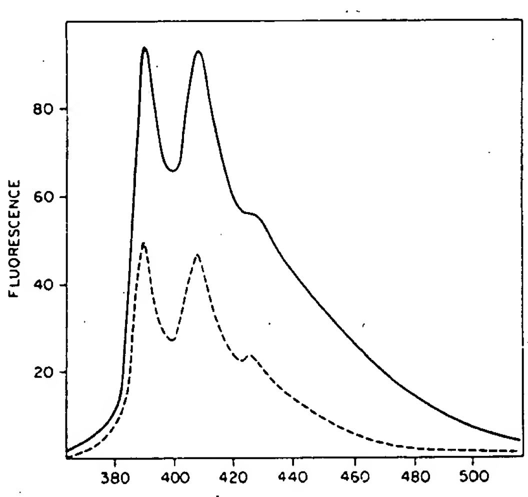

FIG. 6. **Fluorescence emission spectra of PySA-labeled p-25 and gp-30.** Both protein and glycoprotein were isolated as described under "Methods." Excitation was at 346 nm.

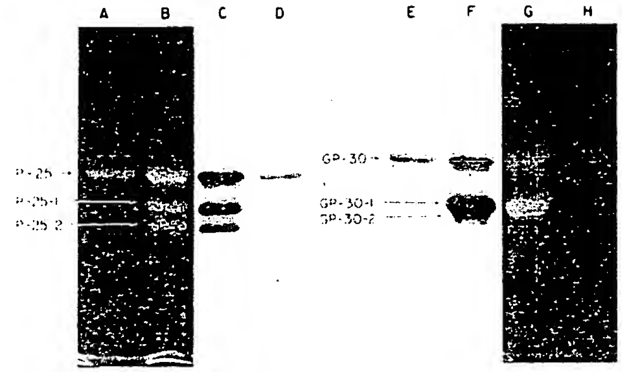

FIG. 7. **SDS-polyacrylamide gel electrophoresis of tryptic fragments of purified p-25 and gp-30.** The samples were treated, and the electrophoresis was performed as described for Fig. 1 on a 12–23% linear gradient gel. Lanes *A*, *B*, *G*, and *H* corresponded to gel visualized under long wavelength UV light. Lanes *C*–*F* correspond to the same gel stained with Coomassie blue. *A*, undigested p-25; *B*, trypsin-digested p-25; *C*, trypsin-digested p-25; *D*, undigested p-25; *E*, undigested gp-30; *F*, trypsin-digested gp-30; *G*, trypsin-digested gp-30; *H*, undigested gp-30.

trypsin at an enzyme:substrate ratio of 1:25 (10). Nonetheless, both p-25 and gp-30 are cleaved at lysine 122, each giving two peptides. p-25 is cleaved into p-25-1 and p-25-2 which represent the NH_2-terminal 122 amino acids and the carboxyl-terminal 104 residues, respectively. Cleavage of gp-30 provides gp-30-1 and gp-30-2, representing the same polypeptide portions as in cleavage of p-25, except that their resolution by SDS-polyacrylamide gel electrophoresis is less satisfactory due to the presence of the carbohydrate moiety on the gp-30-2 fragment (10). Fig. 7 shows the results obtained from the tryptic hydrolysis of PySA-labeled p-25 and gp-30. Once again, the polypeptide pattern upon digestion with trypsin is not modified by PySA labeling and coincides exactly with previously reported ones for unmodified p-25 and gp-30 (10). PySA fluorescence labeling was observed in both the NH_2-terminal and COOH-terminal tryptic fragments from either protein or glycoprotein.

DISCUSSION

After extensive purification, HBsAg derived from human plasma has been shown to contain two proteins, p-25, the major protein, and gp-30, the glycosylated form of p-25. p-25 and gp-30 have been shown to bear distinctive HBsAg antigenic activities as discrete amino acid sequences (32). These components account for more than 90% of the protein of HBsAg but only about 70% of the total mass of HBsAg. The remaining components of HBsAg, namely the lipids and carbohydrates, have not been characterized, nor has their role with regard to the structure or antigenic activity of HBsAg been elucidated.

We describe in this paper that the lipid composition of exhaustively purified HBsAg, subtype adw, does not vary between different preparations from individual HBsAg carriers or between preparations from the same individual whose plasma was obtained at different times. Therefore, we can conclude that the described features of HBsAg lipids are truly characteristic of HBsAg. HBsAg lipids account for approximately 25% of the HBsAg weight and are exclusively composed of phospholipids, cholesterol, cholesteryl esters, and triglycerides. No detectable amounts of any other lipid components were observed in any preparation. In this regard, HBsAg has been previously reported to contain glycolipids (8) which could presumably represent antigenic determinants, as described for other immunogenic systems (33). However, all

of our preparations were examined for glycolipids, but none were detected.

When compared to normal human serum lipoproteins, HBsAg was found to have some similarities with HDL which, for instance, has approximately the same density and contains a protein (Apo A-I) whose molecular weight is similar to that of HBsAg protein components (34). The major lipid components of HDL are also glycerides (~10%), cholesteryl esters (~20%), free cholesterol (~5%), and phospholipids (~41%) (35). Total cholesterol content of HDL is approximately that of HBsAg, even though the relative proportion of cholesterol to cholesteryl esters is approximately 1:1 for HBsAg and 1:3.5–4 for HDL. HBsAg also contains more phospholipids and less triglycerides than HDL. However, the fatty acid composition of the major lipid classes of HBsAg coincides almost exactly with that described for HDL_{2+3}, even though most serum lipoproteins seem to exhibit this feature as a common property regardless of their other differences (35). Nevertheless, because of the similarity between HDL and HBsAg lipids we examined our purified HBsAg preparations for HDL contamination. Automatic Edman degradation of HBsAg (data not shown) resulted in the liberation of phenylthiohydantoin-Met. Methionine has been demonstrated to be the NH_2-terminal residue of both p-25 and gp-30 (10). There was no detectable phenylthiohydantoin-Asp, which would be the expected derivative of the NH_2-terminal residue of Apo A-I (34), which is the major protein constituent of HDL, or any other amino acid, indicating that if HDL or any other contaminant was present, they were in very minor amounts and below the level of detection. Additionally, the absence of Apo A-II, the second most abundant apoprotein of HDL (34), was shown by SDS-gel electrophoresis. Apo A-II, M_r = ~8500, would have moved ahead of p-25, and no traces of protein were observed within that molecular weight range. The Edman degradation procedure would not have been useful with Apo A-II since it has the NH_2 terminus blocked by pyrrolidonecarboxylic acid (34). Additionally, counterelectrophoresis against anti-HDL revealed an HDL content in several HBsAg preparations which was lower than 2–3% and would not account for significant amounts of lipid in HBsAg preparations.

Choline phosphoglyceride is the major lipid component of HBsAg, and it is composed almost exclusively of $HgCl_2$-stable diacyl derivatives (PC) which contain palmitic (~31%), stearic (~16%), oleic (~18%), and linoleic (~26%) acids as the major fatty acid components. From the positional distribution of the acyl chains obtained by hydrolysis with phospholipase A_2, it seems likely that the main molecular species of PC is 1-palmitoyl, 2-linoleoyl-*sn*-glycero-3-phosphorylcholine, which would account for approximately 50% of the PC population plus other minor species composed of combinations of stearic or oleic acids in the *sn*-1 position with either palmitic or oleic acids in the *sn*-2 position.

In order to provide some initial information on the structural organization of the lipid-protein complex in HBsAg we have used the photoactivatable hydrophobic probe PySA which, once incorporated and irradiated under adequate conditions, should become attached to those portions of the HBsAg protein components in contact with the lipid hydrophobic matrix. PySA has been successfully utilized as a marker for the acetylcholine receptor subunits which are in contact with the membrane hydrophobic matrix (19, 20) and also to identify membrane-associated proteins in vesicular stomatitis virus (18). When the photoactivatable PySA is irradiated following incorporation within HBsAg particles, both p-25 and gp-30 components became labeled by this probe, indicating that they are at least partially buried within the lipid environment. The distribution of the label between p-25 and gp-30 can be estimated by either radioactivity (using [^{3}H]PySA), absorbance scans on unstained gels at 354 nm, or fluorescence measurements on electrophoretically eluted p-25 and gp-30 fractions. By all of these, the stoichiometry of PySA labeling is very similar to the stoichiometry of p-25 to gp-30 obtained from 280-nm scans of unstained gels, suggesting a similar exposure to the lipid matrix for both protein components. Indeed, when the labeled p-25 and gp-30 were digested with trypsin and the fragments separated on SDS-polyacrylamide gels, both the COOH-terminal and the NH_2-terminal tryptic fragments of each were found to be labeled. These results are not unexpected since proteins p-25 and gp-30 have identical amino acid sequences (10) and might be expected to interact with the lipids in the same or similar manner. Also these results are consistent with the fact that there are extensive hydrophobic amino acid sequences in both of the tryptic fragments produced (18). Protection against PySA labeling of HBsAg components by the presence of an excess of bovine serum albumin failed. Indeed, the extent and the pattern of labeling as judged by the absorbance ratio 280:346 nm remained unchanged. Additionally, the PySA photoproducts which were associated with bovine serum albumin exhibited the characteristic absorption spectra of free PySA photoproducts which differ from the spectrum of protein-bound PySA photoproducts. This suggests that those PySA photoproducts which are generated in the aqueous solution fail to react with protein, the reaction apparently being quenched by water. On the other hand we may conclude that the PySA labeling, which we observed in the case of HBsAg, occurred with those PySA photoproducts generated within the hydrophobic environment of the HBsAg particle.

The labeling pattern of HBsAg protein components described above is not the result of any observable modification of the protein arrangement due to the incorporation of PySA or the irradiation procedure. In fact, PySA-labeled HBsAg exhibit no measurable decreased antigenic activity when compared with unlabeled HBsAg samples which would have presumably been lost if protein rearrangement had taken place.

Intact HBsAg particles also exhibit a water-exposed region at residues 122–150 of both p-25 and gp-30. This region appears to be an anchoring site for carbohydrates, and also it has been demonstrated that it contains amino acid sequences characteristic for adw and ayw subtypes.[2] These results and the above-described PySA labeling pattern are compatible with a model for HBsAg whereby regions within both the COOH-terminal and NH_2-terminal portions of p-25 and gp-30 are buried within the lipid matrix, while the antigenically important 122–150 region is sticking out of the HBsAg particle, thus exposed to the aqueous surroundings. The identification of those specific amino acids which comprise the regions in contact with lipid cannot be determined from these studies. However, the use of additional methods to cleave the protein into smaller fragments may allow such specific assignments. Such experiments are currently in progress.

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(54) Title: COMBINED VACCINES COMPRISING HEPATITIS B SURFACE ANTIGEN AND OTHER ANTIGENS

(57) Abstract

Stable and effective multivalent vaccine compositions comprising Hepatitis B surface antigen (HBsAg) are described wherein the HBsAg component is stable for one week at 37 °C and is highly immunogenic, for example when the vaccine is administered to infants. The compositions typically comprise HBsAg adsorbed to aluminium phosphate and other antigens, especially those suitable for use in a paediatric vaccine, adsorbed to aluminium phosphate or aluminium hydroxide. Methods for preparing the vaccines and the use of aluminium phosphate to stabilise HBsAg in a multivalent vaccine formulation are also described.

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COMBINED VACCINES COMPRISING HEPATITIS B SURFACE ANTIGEN AND OTHER ANTIGENS.

The present invention relates to novel vaccine formulations, methods for preparing them and to their use in therapy. In particular the present invention relates to novel combination vaccine formulations including a Hepatitis B vaccine component for treating Hepatitis B infections.

Infection with Hepatitis B (HB) virus is a widespread problem but vaccines which have been used for mass immunisation are now available, for example the product 'Engerix-B' (SmithKline Beecham plc). Engerix B has as the Hepatitis B antigenic component Hepatitis B surface antigen (HBsAg) which is obtained by genetic engineering techniques.

However it is often necessary or desirable to administer Hepatitis B vaccine at the same time as other vaccines and this can involve multiple injections. Problems associated with multiple injections include a more complicated administration procedure and a large total injection volume.

There is therefore a need for a combined vaccine comprising a Hepatitis B antigen in combination with other antigens. The other antigens are in particular those capable in a vaccine formulation of preventing Hepatitis A (HA), diphtheria (D), tetanus (T), whole cell pertussis (Pw), acellular pertussis (Pa), Haemophilus influenzae b (Hib) and polio (P).

Aluminium hydroxide (AH) is widely used as an adjuvant in the formulation of vaccines. For example, Engerix B uses Hepatitis B surface antigen (HBsAg) adsorbed to aluminium hydroxide. We have also used AH successfully in the formulation of Hepatitis A vaccine and in the combined vaccines DT, DTPw and DTPa. However, when AH -adsorbed HBsAg is used in combination with other vaccines in a combined formulation there is a substantial decrease of the immune response to HBsAg, resulting in lower or insufficient seroprotection after vaccination. In addition the stability of the HBsAg component of the combined vaccine is poor.

Aluminium phosphate (AP) adsorbed HBsAg has been used in a commercially available monovalent vaccine (HEPPACINE) made by Korean Cheil Sugar Co Ltd. We have found that there is no significant difference in immunogenic properties between an AH- adsorbed HBsAg monovalent vaccine (Engerix B) and an AP-adsorbed HBsAg monovalent vaccine.

European patent application publication number 0 339 667 discloses a bivalent vaccine comprising HBsAg and a Hepatitis A antigen in which either aluminium hydroxide or aluminium phosphate is used as adjuvant. There appears, however, to be no appreciation of the need to avoid aluminium hydroxide as an adjuvant for a multivalent vaccine comprising HBsAg. Furthermore there appears to be no disclosure in this document or elsewhere of a bivalent or multivalent hepatitis B vaccine in which at least one antigen other than HBsAg is adsorbed on aluminium hydroxide and the HBsAg is adsorbed on aluminium phosphate.

Indeed there appears to be no prior enabling disclosure of a stable and effective multivalent vaccine comprising HBsAg at all.

In one aspect the present invention provides a combined vaccine composition comprising Hepatitis B surface antigen (HBsAg) and a number (n) of other antigens in combination with an adjuvant comprising one or more aluminium salts in which the value of n is 1 or greater and in which the adjuvant used to adsorb the HBsAg is not aluminium hydroxide, with the proviso that when n is 1 the other antigen is not an antigen against Hepatitis A.

Preferably n is 2, 3, 4, 5 or 6.

The advantage of the invention is that no substantial decrease in the immunogenicity of the HBsAg occurs in the combined vaccine formulation. Avoiding the use of AH to adsorb the HBsAg component in the vaccine formulation also gives rise to a product of markedly superior stability. A further advantage of the invention is that the aforesaid problems associated with multiple injections are overcome or at least mitigated and a stable, highly immunogenic combined formulation is provided. The compositions of the invention are particularly suitable for administration to children.

Preferably the HBsAg is adsorbed on AP. In particular we have found in human clinical studies that when AP-adsorbed HBsAg is combined with one or more AH-adsorbed or AP-adsorbed antigens in a combined vaccine no substantial decrease in immunogenicity occurs. The stability of the AP-adsorbed HBsAg in the formulation is also greater than AH-adsorbed HBsAg.

Accordingly in a further aspect there is provided a vaccine composition according to the invention in which at least one of the antigens other than HBsAg is adsorbed to aluminium phosphate.

In a further preferred aspect at least one of the antigens other than HBsAg is adsorbed to AH.

In a further aspect, the invention provides a combined vaccine comprising Hepatitis B surface antigen (HBsAg) adsorbed to AP and an antigen adsorbed to AP or to AH selected from an antigen providing immunity against one or more of the following viruses: diphtheria (D); tetanus (T); pertussis (P); Inactivated Polio (IPV); Haemophilus influenzae b (Hib); and Hepatitis A (HA).

In a paediatric vaccine other compatible antigens may also be included, eg antigens known to be effective against meningitis B, meningitis A and C, and otitis media.

As used herein the term 'bivalent' is used to refer to a vaccine comprising a combination of two antigens in total (including HBsAg). The term 'multivalent' is applied to a vaccine composition comprising more than two antigens, for example three, four or five or six antigens.

The meaning of the terms 'aluminium phosphate' and 'aluminium hydroxide' as used herein includes all forms of aluminium hydroxide or aluminium phosphate which are suitable for adjuvanting vaccines.

For example, aluminium phosphate can be a precipitate of insoluble aluminium phosphate (amorphous, semi-crystalline or crystalline), which can be optionally but not exclusively prepared by mixing soluble aluminium salts and phosphoric acid salts. "Aluminium hydroxide" can be a precipitate of insoluble (amorphous, semi-crystalline or crystalline) aluminium hydroxide, which can be optionally but not exclusively prepared by neutralizing a solution of aluminium salts. Particularly suitable are the various forms of aluminium hydroxide and aluminium phosphate gels available from commercial sources for example, Alhydrogel (aluminium hydroxide, 3% suspension in water) and Adju-fos (aluminium phosphate, 2% suspension in saline) supplied by Superfos (Vedbaek, 2950 Denmark).

It will be appreciated that for the first time we are able to provide a stable and effective multivalent vaccine composition comprising HBsAg.

Accordingly, in a further aspect of the invention there is provided a stable and effective combined vaccine composition directed to the prevention of more than two diseases, comprising HBsAg and at least two other antigens.

As regards choice of adjuvant, excellent results are obtained when the HBsAg is adsorbed on AP and at least one of the antigens other than HBsAg is adsorbed to AH. Other suitable adjuvants may, however, be used. For example one or all of the antigens other than HBsAg may be adsorbed to AP.

Preferred stable combination vaccines according to the invention are

Diphtheria-Tetanus- Pertussis-Hepatitis B (DTP-HB)
Diphtheria-Tetanus-Hepatitis B (DT-HB)
DTP - IPV (inactivated polio vaccine) - Hepatitis B

It will be appreciated that for a vaccine containing a Hib component the Hib antigen may be used extemporaneously by formulating the vaccine just prior to administration. In this way the following combined vaccine compositions within the scope of the invention may, for example, be prepared:

Hib-Hepatitis B
DTP-Hib-Hepatitis B
IPV - DTP-Hib-Hepatitis B.

More specifically particular vaccines within the scope of the invention are:

Diphtheria - Tetanus - Pertussis (DTP adsorbed on AH or AP) - Hepatitis B (HBsAg adsorbed on AP)

Diphtheria - Tetanus (DT adsorbed on AP or AH) - Hepatitis B (HBsAg adsorbed on AP).

By 'stable' as used herein to describe a vaccine according to the invention is meant a vaccine which can be kept a 37°C for one week without any substantial loss of immunogenicity of the HBsAg component.

By 'effective' as used herein is meant a vaccine composition, characterised in that the immunogenicity of the HBsAg in the combined vaccine is such that a geometric mean titre of at least 200 mIU/ml, preferably 300 mIU/ml or greater, is found in human infants one month after the third dose of the vaccine when the vaccine is administered at one month intervals in an appropriate vaccination schedule.

In a further aspect the invention provides a multivalent vaccine composition comprising HBsAg and a stabilising adjuvant selected such that the vaccine can be kept at 37° C for one week without any substantial loss in immunogenicity of the HBsAg component. Preferably the multivalent vaccine composition is further characterised by giving rise to a geometric mean titre of at least 200 mIU/ml (one month post third dose), preferably 300 mIU/ml or greater, in human infants when the vaccine is administered at one month intervals in an appropriate vaccination schedule.

As used herein the term 'appropriate vaccination schedule' means a schedule known to those of skill in the art for administering a course of doses of a vaccine, especially for paediatric doses. A schedule of 3, 4 and 5 months may for example be used. This is particularly appropriate for example for DTP - HBsAg containing vaccines according to the invention.

In one aspect the HBsAg can be adsorbed to an aluminium salt other than aluminium hydroxide. Preferably it is adsorbed to AP. The other antigens in the multivalent vaccine formulation may be adsorbed to AP or AH (or both) and are advantageously adsorbed to AH as shown in the examples hereinbelow.

Advantageously the vaccine formulation according to the invention comprises a pertussis vaccine.

The pertussis component is suitably a whole cell pertussis vaccine or an acellular pertussis vaccine containing partially or highly purified antigens.

The above combinations may optionally include a component which is protective against Hepatitis A, i.e. an HAV antigen.

Advantageously the Hepatitis B combination vaccine is a paediatric vaccine.

The preparation of the antigens and adsorption procedure with the adjuvants are well known in the art, see for example as given below.

The preparation of Hepatitis B surface antigen (HBsAg) is well documented. See for example, Harford *et al.(1983)* in Develop. Biol. Standard 54, page 125 , Gregg *et al.* (1987) in Biotechnology 5, page 479, EP A-0 226 846, EP A- 0 299 108 and references therein.

As used herein the expression 'Hepatitis B surface antigen' or 'HBsAg' includes any HBsAg antigen or fragment thereof displaying the antigenicity of HBV surface antigen. It will be understood that in addition to the 226 amino acid sequence of the HBsAg S antigen (see Tiollais et al, Nature, 317, 489 (1985) and references therein) HBsAg as herein described may, if desired, contain all or part of a pre-S sequence as described in the above references and in EP-A- 0 278 940. In particular the HBsAg may comprise a polypeptide comprising an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L-protein of HBsAg relative to the open reading frame on a Hepatitis B virus of ad serotype (this polypeptide is referred to as L*; see EP 0 414 374). HBsAg within the scope of the invention may also include the preS1-preS2 -S polypeptide described in EP 0 198 474 (Endotronics) or analogues thereof such as those described in EP 0 304 578 (Mc Cormick and Jones). HBsAg as herein described can also refer to mutants, for example the 'escape mutant' described in WO 91/14703 or European Patent Application Publication Number 0 511 855 A1, especially HBsAg wherein the amino acid substitution at position 145 is to arginine from glycine.

Normally the HBsAg will be in particle form. The particles may comprise for example S protein alone or may be composite particles, for example (L*,S) where L* is as defined above and S denotes the S-protein of HBsAg. The said particle is advantageously in the form in which it is expressed in yeast.

Suitable antigens for use in vaccines according to the invention are already commercially available and details may be obtained from the World Health Organisation. For example the IPV component may be the Salk inactivated polio vaccine. The pertussis vaccine may comprise a whole cell product , an acellular product or a recombinantly produced product. In particular the pertussis component can be PT (pertussis toxins) or subfractions thereof, FHA (filamentous haemagglutinin antigen), agglutinogens (fimbrial) and outer membrane proteins, including the 69kDa protein (pertactin, non fimbrial agglutinogen). *References*: Robinson, A., Irons, L. I. & Ashworth, A. E., Vaccines, 3, 1985, 11-22; and

Brennan, H. J., Li, S. M., Cowell, J. L., Bishen, M. E., Steven, A. C. Novotny., P, Manclarck, C. R., Infection and Immunity, 56, 1988, 3189-3195.

The component affording protection against Hepatitis A is preferably the product known as 'Havrix' (SmithKline Beecham Biologicals) which is a killed attenuated vaccine derived from the HM-175 strain of HAV [see 'Inactivated Candidate Vaccines for Hepatitis A' by F.E. Andre, A. Hepburn and E.D'Hondt (1980), Prog. Med. Virol. Vol 37, pages 72-95 and the product monograph 'Havrix' published by SmithKline Beecham Biologicals (1991).

Flehmig et al (loc cit., pages 56-71) have reviewed the clinical aspects, virology, immunology and epidemiology of Hepatitis A and discussed approaches to the development of vaccines against this common viral infection.

As used herein the expression 'HAV antigen' refers to any antigen capable of stimulating neutralising antibody to HAV in humans. The HAV antigen preferably comprises inactivated attenuated virus particles or may be, for example an HAV capsid or HAV viral protein, which may conveniently be obtained by recombinant DNA technology.

Vaccine preparation is generally described in New Trends and Developments in Vaccines (1978), edited by Voller *et al.*, University Park Press, Baltimore, Maryland U.S.A.

The amount of antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending on which specific immunogens are employed. Generally it is expected that each dose will comprise 1-1000 μg of total immunogen, preferably 2-100 μg, more preferably 1 - 40 μg, most preferably 1 - 5 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. A primary vaccination course may include 2 or 3 doses of vaccine, given one to two months apart, following the WHO recommendations for DTP immunization.

The invention thus provides a method of preventing hepatitis B and other infections in humans, especially infants, which method comprises treating a human subject in need thereof with an immunologically effective dose of a vaccine according to any aspect of the invention as hereinabove described.

In a further aspect of the invention there is provided a vaccine composition according to the invention for use in medicine.

In a further aspect of the invention there is provided the use of HBsAg for the manufacture of a combination vaccine according to the invention for the prophylaxis of Hepatitis B viral infections.

In a further aspect the invention provides the use of AP for the purpose of acting as a stabiliser for, and/or to maintain the efficacy of, HBsAg in a multivalent vaccine according to the invention.

Specifically the invention provides the use of aluminium phosphate for the purpose of preparing a stable combined vaccine comprising HBsAg and at least one other antigen (preferably at least two other antigens) whereby the stability and/or immunogenicity of the HBsAg component is greater than in the corresponding combined vaccine in which the HBsAg component is adsorbed on AH.

More specifically the invention provides the use whereby the vaccine can be kept at 37° C for 1 week (i.e. 7 days) without substantial loss of immunogenicity of the HBsAg.

Also provided is the use whereby the geometric meant titre (GMT) found one month after the third dose of a course of vaccinations given at one month intervals in an appropriate vaccination schedule to human infants is greater than 200, preferably greater than 300, mIU/ml.

In a further aspect of the present invention there is provided a method of manufacture of a combined (i.e. bivalent or multivalent) vaccine effective in preventing hepatitis B infection as illustrated in the examples hereinbelow.

In one preferred aspect the antigens other than HBsAg are all adsorbed on AH. A very effective DTPa - Hepatitis B vaccine can, for example, be made in this way.

In general, the combined vaccine compositions according to any aspect of the invention can be prepared as follows. The required DT, DTPw, DTPa, HA or other components are adsorbed onto a suitable adjuvant, especially AH or AP; HBsAg is adsorbed onto a suitable stabilising adjuvant, selected as hereinabove described,

especially an aluminium salt other than AH. Preferably it is adsorbed onto AP. After allowing time for complete and stable adsorption of the respective components, the different components are combined under appropriate conditions.

It will be appreciated that certain components, for example the DT, DTPw and DTPa components can be combined separately before adding the adsorbed HBsAg component. Multivalent vaccines comprising HBsAg and other or additional antigens to those mentioned hereinabove may be prepared in a similar manner.

In a preferred aspect there is provided a method of preparing a combined vaccine composition according to the invention wherein the method comprises mixing aluminium phosphate - adsorbed HBsAg with one or more aluminium hydroxide or aluminium phosphate adsorbed antigens.

The following examples illustrate the invention.

EXAMPLES 1-5

Formulations

Particular formulations according to the present invention were prepared as described below.

Example 1 HBsAg adsorption on $AlPO_4$ as concentrate for formulation of combined vaccines.

A suspension of aluminium phosphate containing 0.03 to 0.3 g aluminium (as aluminium phosphate) in isotonic saline, is mixed with a HBsAg concentrate, containing 10 mg HBsAg protein, in a final volume of 10 to 100 ml. After adjusting the pH to 5 - 6.5 the mixture is left 10 - 24 hrs at room temperature with stirring. Antiseptic is then optionally added (i.e. merthiolate, 1 : 20,000 to 1 : 10,000 or 2-phenoxyethanol, 3 to 6 mg/ml) and the volume is brought to 50 ml with isotonic saline.

Example 2 Formulation of combined Diphtheria-Tetanus-Hepatitis B vaccine.

A concentrate containing 25,000 Lf of diphtheria toxoid and 10,000 Lf of tetanus toxoid adsorbed to 0.35 g Al (as aluminium hydroxide or aluminium phosphate) is prepared in a final volume of 0.15 1 of isotonic saline and adjusted to between pH 6 and 7, as specified by WHO for DT and DTP vaccines. This concentrate is combined with 0.05 1 of the Hepatitis B concentrate of example 1.

This mixture is brought to a final volume of 0.5 1 with isotonic saline. Antiseptic media (c.c. merthiolate 1 : 20,000 to 0 : 10,000 or 2-phenoxyethanol, 3 to 6 mg/ml) can be optionally added. The final pH is between 6 and 7, as specified by WHO for DT and DTP vaccines.

One 0.5 ml dose of this bulk vaccine contains, as active ingredients:

D toxoid:	25Lf,
T toxoid:	10 Lf,
HBsAg:	10 μg protein

The procedure can be optionally amended to use higher or lower quantities of the active ingredients.

Example 3 Formulation of combined Diphtheria - Tetanus - pertussis (whole cell vaccine) - Hepatitis B vaccine

A concentrate ex Behringwerke containing 7,500 Lf of diphtheria toxoid, 3,250 Lf of Tetanus toxoid and 15,000 capacity units of *B. pertussis* antigen adsorbed to 0.45 mg Al (as aluminium hydroxide and aluminium phosphate) is prepared in a final volume of 0.4 l of isotonic saline and adjusted to pH 6 - 7, as specified by WHO for DTP vaccines. This concentrate is combined with 0.05 l of Hepatitis B concentrate of example 1.

This mixture is brought to a final volume of 0.5 l with isotonic saline. Antiseptic media (c.c. merthiolate 1 : 20,000 to 0 : 10,000 or 2-phenoxyethanol, 3 to 6 mg/ml) can be optionally added. The final pH is between 6 and 7, as specified by WHO for DT and DTP vaccines.

One 0.5 ml dose of this bulk vaccine contains, as active ingredients:

D toxoid:	7.5Lf,
T toxoid:	3.25 Lf
Pw antigen:	15OU
HBsAg:	10 μg protein.

The procedure can be optionally amended to use higher or lower quantities of the active ingredients.

Example 4 Formulation of Diphtheria-Tetanus-Pertussis (acellular component) vaccine.

A concentrate containing 25,000 Lf of diphtheria toxoid and 10,000 Lf of tetanus toxoid adsorbed to 0.35 g Al (as aluminium hydroxide or phosphate gel) is prepared

in a final volume of 0.15 l of isotonic saline and adjusted to between pH 6 and 7, as specified by WHO for DTP vaccines. 25 mg of inactivated pertussis toxin (DTPa), 25 mg of filamentous hemagglutinin (FHA) and optionally 8 mg of 69kDa outer membrane protein (pertactin), each combined with 0.05 g Al (as aluminium hydroxide or aluminium phosphate) are added. The *B. pertussis* antigens PT, FHA and pertactin can be prepared as described by methods known in the art, for example European patent application 427 462, *PCT application WO 91/12020* or by other procedures giving physiologically acceptable and potent *B. pertussis* antigens.

This mixture is brought to a final volume of 0.5 l with isotonic saline. Antiseptic media (c.c. merthiolate 1 : 20,000 to 0 : 10,000 or 2-phenoxyethanol, 3 to 6 mg/ml) can be optionally added. The final pH is between 6 and 7, as specified by WHO for DT and DTP vaccines.

One 0.5 ml dose of this bulk vaccine contains, as active ingredients:

D toxoid:	25 Lf,
T toxoid:	10 Lf,
DTd toxoid:	25 µg,
FHA toxoid:	25 µg,
69kDa OMP:	8 µg (optional)

The procedure can be optionally amended to use higher or lower quantities of the active ingredients.

Example 5 Formulation of combined Diphtheria - Tetanus - Pertussis (acellular component) - Hepatitis B vaccine

The procedure of example 4 is applied, with the exception that an additional 50 ml of HBsAg adsorbed concentrate as prepared in example. 1 is added to the final mixture.

The resulting mixture is brought to a final volume of 0.5 l with isotonic saline. Antiseptic media (c.c. merthiolate 1 : 20,000 to 0 : 10,000 or 2-phenoxyethanol, 3 to 6 mg/ml) can be optionally added. The final pH is between 6 and 7, as specified by WHO for DT and DTP vaccines.

One 0.5 ml dose of this bulk vaccine contains, as active ingredients:

D toxoid:	25 Lf,
T toxoid:	10 Lf
PTd toxoid:	25 μg,
FHA toxoid:	25 μg,
69kDaOMP:	8 μg (optional).

The procedure can be optionally amended to use higher or lower quantities of the active ingredients.

EXAMPLES 6-10

Animal and Human Studies

Example 6 Formulation of combined Hepatitis A - Hepatitis B vaccines

An inactivated Hepatitis A virus concentrate (460,000 Elisa units), adsorbed to 0.02 to 0.2 g, preferably 0.04 - 0.1 g aluminium (as aluminium hydroxide) in a final volume of about 125ml was combined to 50 ml of concentrate containing 10 mg HBsAg adsorbed to aluminium phosphate as described in example 1.

The resulting mixture was supplemented with isotonic saline and an amino acid concentrate (Travasol, Baxter-Travenol Inc) to obtain a final volume of 0.5 l containing 1.5 g amino acids. The resulting pH was between 6 and 7.

Our 1 ml dose of this bulk vaccine contains, as active ingredients:

Hepatitis A virus antigen:	800 Elisa units
HBsAg:	20 μg protein

The procedure can be optionally amended to use higher or lower quantities of the active ingredients.

Results:

Clinical studies comparing aluminium hydroxide (AH) and aluminium phosphate (AP) adsorbed HBsAg (Monovalent vaccine)

Initially seronegative healthy adult volunteers were immunised with 3 doses of 20 µg HBsAg protein given at one month interval. Antibody levels were determined in sera obtained one month post 2 and 3 doses using the Ausab (Abbott) test. Responses were defined as subjects with titres significantly above background. Titres were expressed in mIU/ml.

Results are expressed as Geometric Mean Titres (GMT) in mIU/ml.

HBsAg Lot	Adjuvant	N.Subj.	Post 2, month 2		Post 3, month 3	
			GMT	% responders	GMT	% responders
100	AH	43	32	86	141	100
101	AH	45	26	93	198	98
102	AH	46	30	84	147	93
105/P	AP	7	43	83	380	100

HBsAg Lot	Adjuvant	N.Subj.	Post 2, month 2		Post 3, month 3	
			GMT	% responders	GMT	% responders
102	AH	51	14	82	126	98
103	AH	50	15	83	110	98
102	AH	54	17	83	133	96
104/P	AP	54	18	96	270	98
105/P	AP	51	14	90	156	96

Example 7

Mouse immunogenicity tests and results of accelerated stability tests for combination vaccines comprising HBsAg with aluminium hydroxide (AH) or aluminium phosphate (AP) as adjuvant

Groups of 10 OF1 mice were immunised subcutaneously with 2 doses of 2.5 µg HBsAg (single component or combined) at days 0 and 14. Blood was drawn off at day 21 and titrated for anti-HBsAg using the Ausab (Abbott) test. Antibody titres were calculated in mIU/ml. The number of responding animals was defined as the number of those with antibody levels significantly above background values. The geometric mean titres was also calculated (GMT).

The results of DT-HB, DTPw-HB, DTPa-HB show that AP adsorbed HBsAg performed better than AH adsorbed HBsAg both in terms of number of responding animals and GMTs. The response to AP adsorbed HBsAg in the combination was comparable to that obtained by monovalent HBsAg administration.

Vaccine	4°C		7 days, 37°C		7 days, 45°C	
	N.resp.	GMT	N.resp.	GMT	N.resp	GMT
Engerix B(HB+AH)	7/10	30	9/10	17	6/10	2.7
Engerix B(HB+AH)	9/10	54	8/10	13	5/10	6
HB (AH)	9/10	45	10/10	55	9/10	32
HB (AP)	9/10	54	10/10	50	7/10	6.9
DTPw(AH)HB(AH)	4/10	1.4	nd	nd	nd	nd
DTPw(AH)HB(AP)	9/10	52	8/10	16	8/10	26
DT(AH)HB(AH)	6/10	1.7	nd	nd	nd	nd
DT(AH)HB(AP)	8/10	44	9/10	21	10/10	36
DTPa(AH)HB(AH)	5/10	1.7	nd	nd	nd	nd
DTPa(AH)HB(AP)	10/10	18	8/10	8	9/10	24

nd: not tested

Example 8

Immunogenicity of HBsAg combined to DTPw in monkeys
Results of aluminium hydroxide (AH) and aluminium phosphate (AP) adsorbed antigen

Cercopithenus aethiops monkeys received two injections of 10 µg HBsAg (alone or combined) at days 0 and 30. Sera were withdrawn at days 30 and 57 and titrated (Ausab, Abbott) for anti-HbsAg. Animals with antibody levels significantly above background (pre-vaccination sera) were considered responders. GMT were calculated in mIU/ml.

Results show AP adsorbed HBsAg performed better than AH adsorbed HBsAg.The response was comparable to that obtained by monovalent HBsAg administration.

	Post 1, day 30		Post 2, day 57	
Vaccine	N. resp.	GMT	N. resp.	GMT
Engerix B (HB)(AH)	4/5	10	5/5	666
DTPw(AH)HB(AH)	4/5	20	5/5	31
DTPw(AH)HB(AP)	5/5	12	5/5	414

Example 9

Clinical studies with combined DTPw vaccines using HBsAg adsorbed to aluminium hydroxide (AH) or aluminium phosphate (AP)

Subjects were immunised with 3 doses of 0.5 ml containing DTPw and 10µg HBsAg protein given at the age of 3, 4 and 5 months. Bleeding was at 6 months and sera were titrated with the Ausab test. Percentage responders (seroconversion) relates to subjects with antibody levels significantly above background. Percentage protection relates to subjects with titres equal to or greater than 10 mIU/ml. GMT in mIU/ml.

Results for DTPw-HB show AP adsorbed HBsAg produced a satisfactory response as opposed to AH adsorbed HBsAg. Seroconversion rates and GMT were comparable to data typically seen with monovalent HBsAg vaccine (Engerix B).

Vaccine	N. subj.	Bleeding Time	% resp.	% prot.	GMT
DTPw(AH).HB(AH)	32	post 2	nd	nd	nd
		post 3	94	84	38.5
DTPw(AH).HB(AP)	29	post 2	97	97	63
	17	post 3	100	100	469

Example 10

Immunogenicity and stability of HBsAg adsorbed to aluminium hydroxide (AH) or aluminium phosphate (AP) in a hepatitis A-Hepatitis B combined vaccine

Groups of 10 OF1 mice were immunised subcutaneously with 2 doses of 2.5 µg HBsAg (single component or combined) at days 0 and 14. Blood was drawn at day 21 and titrated for anti-HBsAg as in Example 7.

Results for immunogenicity and stability of HA-HB combined product showed AP adsorbed HBsAg produced higher antibody levels and a more stable form.

Vaccine	Exposure	N. resp.	GMT
HA(AH).HB(AH)	4°C	9/10	41
	1 month, 37°C	6/10	5.6
	1 month, 45°C	5/10	6.4
HA(AH).HB(AP)	4°C	10/10	80
	1 month, 37°C	9/10	45
	1 month, 45°C	8/10	18
Engerix B HB(AH)	4°C	8/10	58

Example 11: Further Clinical Results in humans

1. Immunogenicity of DTPw - Hepatitis B vaccines in infants

Experiment A

Studies in Slovakia: Schedule: 3-4-5 months. 10µg HBsAg; DTPw ex Behringwerke (DT on AH; Pw on a mixture of AH and AP)

Anti-Hbs titres

HBsAg adjuvant	Time	N	GMT	SP(%)
Hydroxide	Post II (5 months)	44	45	79.5
Hydroxide	Post III (6 months)	13	34	69.2
Phosphate	Post II (5 months)	32	80	97.0
Phosphate	Post III (6 months)	32	396	100

In this and other examples Post II means after the second dose, post III after the third dose. GMT is always measured one month after the injection time shown in the schedule. SP is the seroprotection rate.

Anti-Diphtheria, Tetanus, B pertussis titres

Post III results	N	GMT	%>0.1 IU/ml	GMT Post/Pre
Anti-Diphtheria	38	2.302	100	37.4
Anti-Tetanus	38	3.281	100	38.4
Anti-B pertussis	38	61	-	7.7

Experiment B

Study in Greece: Schedule 2-4-6 months (same vaccine as for Experiment A)

Anti-HBs titres (interim results)

HBsAg adjuvant	Time	N	GMT	SP(%)
Hydroxide	Month 7	22	284	90.5
Hydroxide	Month 7	17	193	94.4
Phosphate	Month 7	23	1794	92.0

Experiment C

Study in Slovakia: Schedule 3-4-5 (HBsAg =5µg on aluminium phosphate; DTP ex Behringwerke as for Experiment A)

Anti-HBs titres

Time	N	GMT	SP(%)
Post II	21	94	90.5
Post III	18	311	100

Experiment D

Study in Slovakia: Schedule 3-4-5 months of age (HBsAg =10µg on aluminium phosphate; DTPw ex Behringwerke as for Experiment A)

Anti-HBs titres

Time	N	GMT	SP(%)
Pre	24	0	0
Post II (month 5)	13	259	92.3
Post III (month 6)	10	592	100.0

Anti-diphtheria antibodies

Timing	N	GMT	SP (%)	GMT Post/Pre
Pre	32	0.054	6.3	1.0
Post II	16	1.094	93.8	20.4
Post III	11	2.314	100.0	43.1

Anti-tetanus antibodies

Timing	N	GMT	SP (%)	GMT Post/Pre
Pre	32	0.083	34.4	1.0
Post II	16	3.146	100.0	37.9
Post III	11	7.989	100.0	96.4

Anti-B pertussis antibodies

Timing	N	GMT	GMT Post/Pre
Pre	32	8	1.0
Post II	16	20	2.7
Post III	11	50	6.6

2. Immunogenicity of DTPa - Hepatitis B vaccines in infants

Experiment A

Study in Turkey. HBsAg 10µg on AP; DTP (acellular) on AH. Preliminary results

Group 1 (DTPa - Engerix B combination)

Timing	N	S+	%	GMT
Pre	19	0	0	0
Post I	19	4	21.1	24
Post II	19	18	94.7	146
Post III	19	19	100.0	345

Group 2 (DTPa plus Engerix B; separate simultaneous injections)

Timing	N	S+	%	GMT
Pre	8	0	0	0
Post I	8	2	25.0	37
Post II	8	5	62.5	33
Post III	7	6	83.7	385

Key: N = number of subjects tested; S+ = number of subjects seropositive at a given blood sampling time; % = seroconversion rate and GMT = geometric mean antibody titre of seroconverters

CLAIMS

1. A combined vaccine composition comprising Hepatitis B surface antigen (HBsAg) and a number (n) of other antigens in combination with an adjuvant comprising one or more aluminium salts in which the value of n is 1 or greater and in which the adjuvant used to adsorb the HBsAg is not aluminium hydroxide, with the proviso that when n is 1 the other antigen is not an antigen against Hepatitis A.

2. A vaccine composition as claimed in claim 1 in which the HBsAg is adsorbed to aluminium phosphate.

3. A vaccine composition as claimed in claim 2 in which at least one of the other antigens is adsorbed to aluminium phosphate.

4. A vaccine composition as claimed in claim 2 in which at least one of the other antigens is adsorbed to aluminium hydroxide.

5. A vaccine composition as claimed in any preceding claim in which n is 2, 3, 4, 5 or 6.

6. A combined vaccine composition according to claim 5 wherein the antigen adsorbed to aluminium hydroxide or aluminium phosphate is selected from an antigen providing immunity against diphtheria (D); tetanus (T); pertussis (P); Inactivated Polio (IPV); Haemophilus influenzae b (Hib) and Hepatitis A (HA).

7. A stable and effective combined vaccine composition directed to the prevention of more than two diseases comprising HBsAg and at least two other antigens.

8. A vaccine composition according to claim 7 in which the HBsAg is adsorbed to aluminium phosphate.

9. A vaccine composition according to claim 8 in which at least one of the antigens other than HBsAg is adsorbed to aluminium hydroxide.

10. A combined vaccine composition as claimed in any one of claims 7 to 9 which is :

Diphtheria-Tetanus-Pertussis (DTP) - Hepatitis B; or

Diphtheria - Tetanus (DT) - Hepatitis B ; or

DTP - IPV (inactivated polio vaccine) - Hepatitis B.

11. A combined vaccine composition according to any previous claim in which the stability of the vaccine is such that the vaccine can be kept at 37° C for 1 week without substantial loss of immunogenicity of the HBsAg component.

12. A combined vaccine composition according to any previous claim, characterised in that the immunogenicity of the HBsAg in the combined vaccine is such that a geometric mean titre of 200 mIU/ml (one month post third dose) or greater is found in human infants when a course of the vaccine is given at one month intervals in an appropriate vaccination schedule.

13. A combined vaccine composition as claimed in any preceding claim comprising an antigen component which is protective against Hepatitis A.

14. A combined vaccine composition according to any preceding claim which comprises a pertussis component.

15. A combined vaccine according to claim 14 in which the pertussis component is the whole cell pertussis vaccine or the acellular pertussis vaccine containing partially or highly purified antigens.

16. A multivalent vaccine composition comprising HBsAg and a stabilising adjuvant, the adjuvant being selected such that the vaccine can be kept at 37° C for one week without any substantial loss in immunogenicity of the HBsAg component.

17. A multivalent vaccine composition according to Claim 16 further characterised in that it gives rise to a geometric mean titre of at least 200 mIU/ml (1 month post third dose) when a course of the vaccine is given to human infants at one month intervals in an appropriate vaccination schedule.

18. A multivalent vaccine according to Claim 16 or 17 in which the adjuvant is selected from one or more aluminium salts with the proviso that the HBsAg component is not adsorbed on aluminium hydroxide.

19. A multivalent vaccine according to Claim 18 in which the HBsAg is adsorbed to aluminium phosphate.

20. A multivalent vaccine according to Claim 18 or 19 in which the antigens present in the vaccine formulation other than HBsAg are adsorbed on aluminium hydroxide.

21. A vaccine formulation according to Claim 20 which is Diphtheria - Tetanus - Perrtussis (acellular) - HBsAg.

22. A multivalent vaccine formulation according to Claim 18 or 19 in which the antigens present in the vaccine formulation other than HBsAg are adsorbed on aluminium phosphate.

23. A vaccine composition according to Claim 22 which is Diphtheria - Tetanus - Pertussis (whole cell) - HBsAg.

24. A composition as claimed in any one of claims 1 to 23 for use in medicine.

25. Use of HBsAg in the manufacture of a combination vaccine according to any one of claims 1 to 23 for the prophylaxis of Hepatitis B viral infections.

26. A method of preparing a combination vaccine composition as claimed in claims 1 to 23 in which the HBsAg is adsorbed to AP wherein the method comprises mixing aluminium phosphate - adsorbed HBsAg with one or more aluminium hydroxide or aluminium phosphate adsorbed antigens.

27. Use of aluminium phosphate as an adjuvant for adsorbing HBsAg characterised in that the use is for the purpose of formulating a stable and effective combined vaccine comprising HBsAg and at least 1 other antigen whereby the stability and /or immunogenicity of the HBsAg component is greater than in the combined vaccine in which the HBsAg component is adsorbed on aluminium hydroxide.

28. Use according to Claim 27 in which the stability of the vaccine is such that the vaccine can be kept at 37° C for 1 week without substantial loss of immunogenicity of the HBsAg.

29. Use according to Claim 27 in which the immunogenicity of the HBsAg in the combined vaccine is such that a geometric mean titre of 200 mIU/ml (one month post dose) or greater is found in human infants after a course of 3 doses of the vaccine given at one month intervals in an appropriate immmunisation schedule.

30. Use according to any one of claims 27 to 29 in which there are at least 2 other antigens in the combined vaccine.

31. A method of preventing hepatitis B infections in humans, which method comprises treating human subjects in need thereof with an effective dose of a vaccine according to any one of claims 1 to 23.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/01276

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K39/295; A61K39/39

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	INFECTION AND IMMUNITY vol. 51, no. 3, March 1986, WASHINGTON US pages 784 - 787 P. COURSAGET ET AL. 'SIMULTANEOUS ADMINISTRATION OF DIPHTERIA-TETANUS-PERTUSSIS-POLIO AND HEPATITIS B VACCINES IN A SIMPLIFIED IMMUNIZATION PROGRAM:...' THE WHOLE ARTICLE	1,5-7, 10,14-15
X	RESEARCH DISCLOSURE no. 329, September 1991, HAVANT GB 'POLYVALENT ANTIGEN VACCINE FOR HUMAN USE' 32975	1,5-7, 10,13-15

-/--

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 AUGUST 1993	14 -09- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	REMPP G.L.E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EP,A,0 339 667 (JURIDICAL FOUNDATION THE CHEMO-SERO THERAPEUTIC RESEARCH INSTITUTE) 2 November 1989 cited in the application see page 2, line 46 - page 4, line 2 see page 5, line 24 - line 36 see page 9, line 20 - page 10, line 39	1-6,13, 20,24,25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 93/01276

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claim 31 is directed to a method of treatment of the human /animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9301276
SA 74347

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0339667	02-11-89	JP-A- 1279842	10-11-89
		US-A- 5151023	29-09-92

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

Nucleotide sequence of the gene coding for the major protein of hepatitis B virus surface antigen

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DNA extracted from hepatitis B virus Dane particles has been cloned in bacteria using a plasmid vector. A full-length clone has been examined by restriction endonuclease analysis, and the nucleotide sequence of an 892-base pair fragment from cloned hepatitis B viral DNA encoding the surface antigen gene is reported. The amino acid sequence deduced from the DNA indicates that the surface antigen is a protein consisting of 226 amino acids and with a molecular weight of 25,398. The portion of the gene coding for this protein apparently contains no intervening sequences.

VIRAL HEPATITIS is a major worldwide public health problem. Its aetiology is associated with at least three distinct viral families: hepatitis A, hepatitis B and non-hepatitis A/hepatitis B (ref. 1). The characterisation of the viruses, the pathology associated with the infection and the development of effective means of control have been severely hampered by the very narrow host range specificity of the viruses, and the inability to replicate the virus and obtain cytopathology in tissue culture systems. Nevertheless, progress has been made in identification and preliminary characterisation of the viruses from the sera of infected individuals and from livers at autopsy. The hepatitis A virus is apparently a picornavirus detected as a 27-nm particle[2,3]. In addition to being infective in humans and chimpanzees, it has recently been transferred to marmosets and has been replicated in tissue culture[4-6].

The plasma of individuals infected with heptatitis B show three major particulate structures containing the antigenic determinants apparently specified by the hepatitis viral genome. These include the predominant 22-nm particles, 22-nm filaments of various lengths, and the 42-nm spherical form known as the Dane particle[7,8]. The Dane particle is probably the hepatitis B virion. Recently, a human hepatoma cell grown in tissue culture was shown to produce small quantities of hepatitis B antigens[9].

In instances where productive infections and high titres of viruses cannot be obtained in alternative hosts or tissue culture cells, recombinant DNA methods can be used to obtain large quantities of the virus genome for characterisation and biological activity studies. Structural analysis of the various antigens may allow identification and sequence determination of the various genes of the virus. This information, plus the availability of the viral genome, will greatly aid the study of the pathology and eventually the development of antiviral therapy. In particular, it may be possible, using segments of the viral genome, to produce appropriate viral antigens in alternative hosts such as bacteria or yeast for the development of a vaccine.

Hepatitis B is a particularly attractive paradigm for the development of this approach. The hepatitis B viral genome is relatively simple, perhaps consisting of only 3,200 bases[10,11]. The surface coat, which can be removed by treatment with detergents, contains two main polypeptides, P1 (22–24,000 MW) and P2 (25–29,500 MW)[12]. These molecules are antigenically indistinguishable and protein components are probably identical. Their amino acid compositions are the same within the limits of the analyses used, and the first 19 residues from the N-terminus and the three amino acid residues at the carboxy-terminus seem to be identical[13]. The higher molecular weight molecule (P2) is a glycoprotein, and it has been suggested that the major virus-specific surface antigens are contained within a single gene. Five to seven other polypeptides ranging upward in size to 97,000 MW have been reported to be present in surface antigen preparations[14]. It is not known whether any or all of these molecules are coded for by the viral genome. Viral core preparations contain a major polypeptide of MW 17,000 to

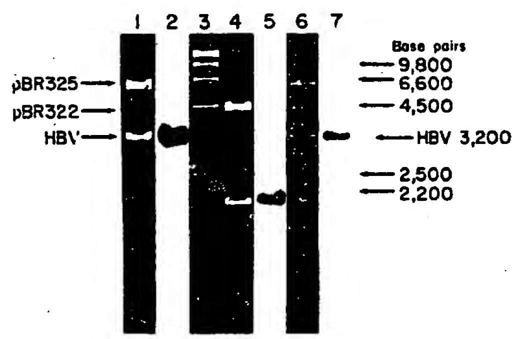

Fig. 1 Cloning and hybridisation of hepatitis B viral DNA. Double-stranded DNA was synthesised in Dane particles (isolated from human sera by Merck Laboratories) by the endogenous DNA polymerase reaction[10]. Incubation was for 3 h at 37 °C in a reaction mixture containing Dane particles (250 ng of DNA), 50 mM Tris-HCl (*p*H 7.5), 0.1 M NaCl, 20 mM $MgCl_2$, 40 mM NH_4Cl, 0.4% NP40, 10 mM 2-mercaptoethanol and 0.2 mM each dATP, dGTP, dCTP and dTTP. DNA was isolated by phenol extraction and ethanol precipitation after digesting the Dane particles for 60 min at 56 °C with proteinase K (0.8 mg ml^{-1}). Dane DNA was digested to completion with endonuclease *Eco*RI (producing a single 3,200-base pair fragment) and with *Bam*HI (yielding two fragments of 2,100 and 1,100 base pairs). Digested Dane DNA (250 ng) was ligated to the appropriate plasmid (50 ng of *Eco*RI digested pBR325 (ref. 18), or 680 ng of phosphatase-treated, *Bam*HI-digested pBR322 (ref. 30)) for 15 h at 14 °C in a reaction mixture containing 50 mM Tris-HCl (*p*H 8), 1 mM ATP, 10 mM $MgCl_2$, 20 mM dithiothreitol and 1 unit of T4 DNA ligase (New England Biolabs). The reaction mixture was used to transform *E. coli* HB101 (in P3/HV1 containment conditions) or *E. coli* χ1776 (in P2/HV2 containment conditions). Recombinant colonies derived from the DNA ligated to the *Bam*HI site of pBR322 were selected by their ampicillin resistance and tetracycline sensitivity[17] and screened for plasmid size[19]. A clone (pHBV-2100) of approximately 6,500 base pairs was identified by a modified toothpick assay[19]. Recombinant colonies derived from the DNA ligated to the *Eco*RI site of pBR325 (ref. 18) were selected by their sensitivity to chloramphenicol. A clone (pHBV-3200) containing plasmid DNA of approximately 8,600 base pairs was identified by the toothpick assay. Supercoiled plasmid DNA was isolated from either clone by a cleared lysate procedure[31], purified by CsCl–ethidium bromide gradient centrifugation[31], digested with *Eco*RI or *Bam*HI and analysed in 1% agarose gels. DNA fragments were hybridised according to Southern[32] to Dane particle DNA labelled by nick translation[33]. Lane 1, ethidium bromide staining of fragments derived from digestion of pHBV-3200 with *Eco*RI. Lane 2, hybridisation of the same fragments to ^{32}P-Dane particle DNA and autoradiography. Lane 3, *Hin*dIII-digested λ DNA as MW standards. Lane 4, ethidium bromide staining of fragments derived from digestion of pHBV-2100 with *Bam*HI. Lane 5, hybridisation of the same fragments to ^{32}P-Dane particle DNA and autoradiography. Lane 6, ethidium bromide staining of fragments derived from digestion of pHBV-64 (a clone of pHBV-3200 viral DNA inserted in the *Pst*I site of pBR322). Lane 7, hybridisation of the same fragments to ^{32}P-Dane particle DNA and autoradiography.

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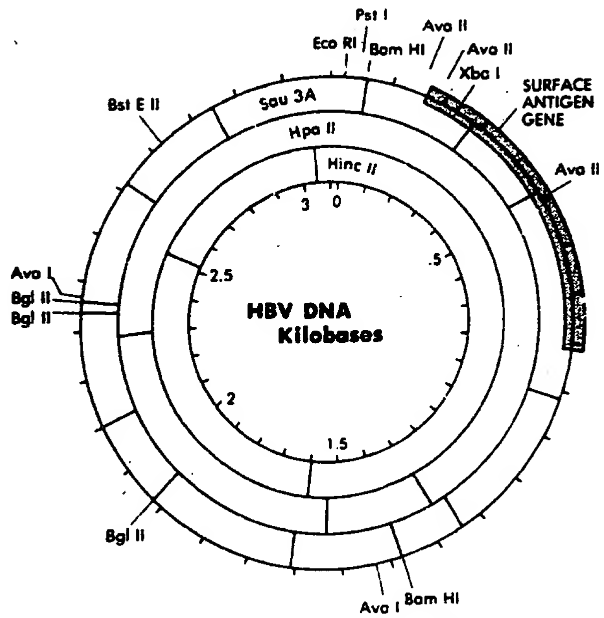

Fig. 2 Restriction endonuclease cleavage map of cloned HBV DNA in plasmid pHBV-3200. Cleavage with restriction endonucleases was carried out in the conditions described by the suppliers with excess amounts of each enzyme. Analysis of the resulting fragments was by electrophoresis in 7% or 10% acrylamide slab gels in 50 mM Tris-borate (pH 8.3) and 1 mM EDTA or in 1-2% agarose slab gels in 50 mM Tris-acetate (pH 8.3) and 1 mM EDTA.

19,000 and a variety of other larger polypeptides of MWs 25,000 to 200,000 (ref. 12). Whether these polypeptides are virus-specific determinants is unknown. A third class of antigens associated with hepatitis infection are the e antigens[15], which recent studies suggest are a dissociated form of the core antigen[16].

Here we report the cloning in bacteria and structural analysis of DNA from hepatitis B virus (HBV). We have defined the location of the viral surface antigen gene in the restriction endonuclease cleavage map of the cloned HBV genome. Nucleotide sequence of an 892-base pair region encoding the surface antigen gene allows us to deduce the complete amino acid sequence of this protein.

Cloning of DNA from hepatitis B virus Dane particles

DNA from Dane particles (prepared from pooled sera received from Merck, Sharp and Dohme) was labelled with ^{32}P-dATP and ^{32}P-dCTP using the endogenous DNA polymerase reaction[10], and purified as described by Landers *et al.*[11]. Several different DNA preparations were cleaved by the restriction endonuclease *Eco*RI into a single fragment of approximately 3,200 base pairs and by *Bam*HI into two fragments of approximately 2,100 and 1,100 base pairs (results not shown). For cloning, the Dane particle DNA was digested with *Bam*HI endonuclease and separately with *Eco*RI, ligated to the respective sites of pBR322 (ref. 17) and pBR325 (ref. 18) and used to transform *Escherichia coli* χ1776. Ampicillin-resistant colonies derived from the DNA ligated into the *Bam*HI site were further screened for their sensitivity to tetracycline[17] and the size of the plasmids they contained[19]. Similarly, recombinant colonies derived from the DNA ligated into the *Eco*RI site of pBR325 were screened for their sensitivity to chloramphenicol[18] and by analysis of their plasmids[19].

Putative recombinant plasmids were isolated and examined by agarose gel electrophoresis after digestion by *Eco*RI and *Bam*HI. One clone from the *Eco*RI experiment (named pHBV-3200) was found to contain a hybrid plasmid with an inserted DNA fragment of approximately 3,200 base pairs, the reported size of linearised viral DNA[20]. A clone from the *Bam*HI experiment (named pHBV-2100) was found to contain a plasmid with a 2,100-base pair insert. The identities of the inserts within pHBV-3200 and pHBV-2100 were verified by cleavage of the plasmids with *Eco*RI and *Bam*HI and hybridisation of the fragments with ^{32}P-labelled Dane particle DNA (Fig. 1). Physical mapping of both inserted fragments with restriction endonucleases showed that pHBV-2100 contains a *Bam*Hi-derived insert also present in pHBV-3200.

Location and sequence analysis of the fragment containing the HBV surface antigen gene

A physical map containing the cleavage sites for several restriction endonucleases was prepared from pHBV-3200. This map has been obtained by a combination of enzyme digestions and DNA sequence analysis (Fig. 2). No restriction sites were found for *Xma*I, *Sst*I, *Kpn*I, *Hpa*I, *Xho*I and *Sac*I endonucleases. The map is similar, but not identical, to those recently described by others for cloned hepatitis viral DNA[21-23]. For example, the map reported by Charnay *et al.*[23] shows an extra *Bam*HI site in the region containing the surface antigen gene. Examination of the nucleotide sequence in this region (Figs 3, 4) shows that the sequence GGATCA coding for the amino acids Gly-Ser may be converted to a *Bam*HI site GGATCC by a change of one base in the third position of the codon, which would therefore not result in a change in the amino acid sequence.

The HBV surface antigen gene was located by extensive nucleotide sequence analysis of the entire cloned viral genome to identify the sequence coding for the 19 amino acids present at the amino-terminus of the protein[13]. The location of the gene within the physical map of the cloned viral DNA is shown in Fig. 2.

The nucleotide sequence of an 892-base pair region encoding this gene was determined as summarised in Fig. 5 legend. The sequence, including the restriction enzyme cleavage sites, is presented in Fig. 3. The translation of this sequence in one of the phases (Fig. 4) predicts precisely the sequence of the 19 N-terminal amino acids of the proteins P1 and P2, except for residue 15, in which the DNA sequence predicts a leucine instead of the serine. Re-examination of the original data of Peterson and Vyas has shown that residue 15 is indeed leucine, and that the original report was incorrect. Furthermore, the amino acid sequencing data up to residue 31 is completely compatible with the amino acid sequence predicted by the DNA, with the exception of residue 24 (arginine), in which insufficient amounts of the phenylthiohydantoin amino acid were formed for analysis (D. Peterson, personal communication). The reported C-terminal sequence, Val-Tyr-Ile[13], is in phase from 204 amino acid residues towards the 3' RNA terminus just before the ochre termination codon, UAA. The polypeptide encoded by these sequences is 226 amino acids long and has a MW of 25,398, in satisfactory agreement with the mass for P1 (22-24,000) determined by SDS gel electrophoresis[12]. The amino acid composition also agrees very closely with that reported for this protein[13]. Note also the relatively high content of proline (10.2%), tryptophan (5.8%), aromatic amino acids (Trp, Tyr, Phe) (15.0%) and hydrophobic amino acids (Val, Ile, Leu) (26.6%).

Because of the prevalence of intervening sequences in eukaryotic genes (see ref. 24 and refs therein), it is not possible to presume the colinearity of a gene with the amino acid sequence of the protein product. However, there is no evidence for an intervening sequence in the surface antigen gene, as the molecule predicted by the DNA sequence closely approximates the characteristics of the isolated surface antigen P1. Any intervening sequence(s) would have to be small (<150 bases); most intervening sequences in structural genes are longer. The N-terminal and C-terminal ends of the molecule are in phase, thus any intervening sequence must also maintain the phase. Furthermore, preliminary studies suggest that the number of major peptides resolved from trypsin-treated aminoethylated

P1 agrees quite well with those predicted from the amino acid sequence derived from the gene (D. Peterson, personal communication). Further studies on P1 or its mRNA should resolve this issue decisively. In the meantime, it seems justified to assume colinearity of the gene with the mRNA for purposes of examination of the primary structure of P1. The high concentration of proline residues and their dispersion throughout the molecule preclude the possibility of a high content of α-helix. Indeed, according to the Fasman rules[25], there is only approximately 4% α-helix. However, there is a significant amount of β form (~30%) and also more than 10% β-turns. Thus, the molecule probably has a globular configuration. Perhaps the most striking primary structural feature of the molecule is a 19-amino acid long hydrophobic region. The sequences flanking this region are particularly rich in proline and cysteine residues. This hydrophobic region may be the site for intermolecular interaction of P1 monomers, and the abundant cysteine residues could make intra- and intermolecular linkages to form the surface coat of the virus. Because of the likelihood that P1 is glycosylated, we searched for possible sites of glycosylation at asparagine residues (Asn-X-Ser/Thr)[26]. There are three such sites at amino acid positions 3, 59 and 146.

Directions for future study

Although P1 and P2 are the major peptides, five or six other proteins of larger size (up to ~100,000 MW) have been observed in surface antigen preparations[12]. One of these, P6 (~72,000 MW), is sometimes present as a major component[12]. This family of molecules cross-reacts antigenically and hence must be related structurally. Whether they represent aggregates of P1/P2 or are distinct molecules is not known. We have not detected nucleotide sequences encoding similar amino acid sequences in the other regions of the viral genome. The possibility that they are formed by processing from a single peptide precursor should also not be overlooked. However, the DNA sequence suggests that it is unlikely that a precursor could extend from the C-terminus because several other termination codons exist in the 3′ RNA direction. Furthermore, the nucleotide sequence which could code for a protein of 190 amino acids begins with the methionine codon AUG, 41 bases after the termination codon of the surface antigen gene (Fig. 5). Screening of the sequence of the entire virus suggested that this may be the gene coding for the major core protein. Further structural analysis of the core protein must be carried out before a

```
BamHI  HinfI            MboII                                                       MnlI
GGATCCCAGAGTCAGGGGTCTGTATCTTCCTGCTGGTGGCTCCAGTTCAGGAACAGTAAACCCTGCTCCGAATATTGCCTCTCACATCTC
CCTAGGGTCTCAGTCCCCAGACATAGAAGGACGACCACCGAGGTCAAGTCCTTGTCATTTGGGACGAGGCTTATAACGGAGAGTGTAGAG
 MboI

        TacI           U1                                          HinfI    U1
GTCAATCTCCGCGAGGACTGGGGACCCTGTGACGAACATGGAGAACATCACATCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCGGG
CAGTTAGAGGCGCTCCTGACCCCTGGGACACTGCTTGTACCTCTTGTAGTGTAGTCCTAAGGATCCTGGGGACGAGCACAATGTCCGCCC
           MnlI    Ava2                                              Ava2

         HindII   HinfI                    HinfI   HinfI                                MboI
GTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCTCCCGTGTG
CAAAAAGAACAACTGTTCTTAGGAGTGTTATGGCGTCTCAGATCTGAGCACCACCTGAAGAGAGTTAAAAGATCCCCCTAGAGGGCACAC
                    MnlI                XbaI

   BalI                    MnlI       Hph     MnlI     MnlI     ApyI
TCTTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCG
AGAACCGGTTTTAAGCGTCAGGGGTTGGAGGTTAGTGAGTGGTTGGAGGACAGGAGGTTAAACAGGACCAATAGCGACCTACACAGACGC
   HaeIII                                                        EcoRII

              MnlI        Bbr         MnlI MboII         MboI                            MnlI
GCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTATTGGTTCTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCC
CGCAAAATAGTATAAGGAGAAGTACGACGACGATACGGAGTAGAAGAATAACCAAGAAGACCTAATAGTTCCATACAACGGGCAAACAGG
                 MboII

      EcoRII MboII          U1                  HinfI                        MnlI
TCTAATTCCAGGATCAACAACAACCAGTACGGGACCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTG
AGATTAAGGTCCTAGTTGTTGTTGGTCATGCCCTGGTACGTTTTGGACGTGCTGAGGACGAGTTCCGTTGAGATACAAAGGGAGTACAAC
       ApyI                 Ava2

                                                    EcoRII                       AsuI MnlI
CTGTACAAAACCTACGGATGGAAATTGCACCTGTATTCCCATCCCATCGTCCTGGGCTTTCGCAAAATACCTATGGAGTGGGCCTCAGT
GACATGTTTTGGATGCCTACCTTTAACGTGGACATAAGGGTAGGGTAGCAGGACCCGAAAGCGTTTTATGGATACCCTCACCCGGAGTCA
                                                    ApyI                         HaeIII

                                                                             AluI
CCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGCTATATGGATGAT
GGCAAAGAGAACCGAGTCAAATGATCACGGTAAACAAGTCACCAAGCATCCCGAAAGGGGGTGACAAACCGAAAGTCGATATACCTACTA

          AsuI                HinfI
GTGGTATTGGGGGCCAAGTCTGTACAGCATCGTGAGTCCCTTTATACCGCTGTTACCAATTTTCTTTTGTCTCTGGGTATACATTTAAAC
CACCATAACCCCCGGTTCAGACATGTCGTAGCACTCAGGGAAATATGGCGACAATGGTTAAAAGAAAACAGAGACCCATATGTAAATTTG
          HaeIII

                                                                                  MboI
CCTAACAAAACAAAAAGATGGGGTTATTCCCTAAACTTCATGGGCTACATAATTGGAAGTTGGGGAACTTTGCCACAGGATC
GGATTGTTTTGTTTTTCTACCCCAATAAGGGATTTGAAGTACCCGATGTATTAACCTTCAACCCCTTGAAACGGTGTCCTAG
```

Fig. 3 Nucleotide sequence of the HBV surface antigen gene and adjacent regions. Sequence analysis was carried out by the method of Maxam and Gilbert[14] as outlined in Fig. 4. The enzyme *MboI* is an isoschizomer of *Sau3A*. U1 indicates Sau_{96I} sites.

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G GAU CCC AGA GUC AGG GGU CUG UAU CUU CCU GCU GGU GGC UCC AGU UCA GGA ACA GUA AAC CCU GCU CCG AAU AUU GCC UCU CAC AUC

                                                1                                   10
                                                met glu asn ile thr ser gly phe leu gly pro leu leu val leu gln ala
UCG UCA AUC UCC GCG AGG ACU GGG GAC CCU GUG ACG AAC AUG GAG AAC AUC ACA UCA GGA UUC CUA GGA CCC CUG CUC GUG UUA CAG GCG

    20                              30                                          40
gly phe phe leu leu thr arg ile leu thr ile pro gln ser leu asp ser trp trp thr ser leu asn phe leu gly gly ser pro val
GGG UUU UUC UUG UUG ACA AGA AUC CUC ACA AUA CCG CAG AGU CUA GAC UCG UGG UGG ACU UCU CUC AAU UUU CUA GGG GGA UCU CCC GUG

    50                              60                                          70
cys leu gly gln asn ser gln ser pro thr ser asn his ser pro thr ser cys pro pro ile cys pro gly tyr arg trp met cys leu
UGU CUU GGC CAA AAU UCG CAG UCC CCA ACC UCC AAU CAC UCA CCA ACC UCC UGU CCU CCA AUU UGU CCU GGU UAU CGC UGG AUG UGU CUG

    80                              90                                          100
arg arg phe ile ile phe leu phe ile leu leu leu cys leu ile phe leu leu val leu leu asp tyr gln gly met leu pro val cys
CGG CGU UUU AUC AUA UUC CUC UUC AUC CUG CUG CUA UGC CUC AUC UUC UUA UUG GUU CUU CUG GAU UAU CAA GGU AUG UUG CCC GUU UGU

    110                             120                                         130
pro leu ile pro gly ser thr thr thr ser thr gly pro cys lys thr cys thr thr pro ala gln gly asn ser met phe pro ser cys
CC" CUA AUU CCA GGA UCA ACA ACA ACC AGU ACG GGA CCA UGC AAA ACC UGC ACG ACU CCU GCU CAA GGC AAC UCU AUG UUU CCC UCA UGU

    140                             150                                         160
cys cys thr lys pro thr asp gly asn cys thr cys ile pro ile pro ser ser trp ala phe ala lys tyr leu trp glu trp ala ser
UGC UGU ACA AAA CCU ACG GAU GGA AAU UGC ACC UGU AUU CCC AUC CCA UCG UCC UGG GCU UUC GCA AAA UAC CUA UGG GAG UGG GCC UCA

    170                             180                                         190
val arg phe ser trp leu ser leu leu val pro phe val gln trp phe val gly leu ser pro thr val trp leu ser ala ile trp met
GUC CGU UUC UCU UGG CUC AGU UUA CUA GUG CCA UUU GUU CAG UGG UUC GUA GGG CUU UCC CCC ACU GUU UGG CUU UCA GCU AUA UGG AUG

    200                             210                                         220                     226
met trp tyr trp gly pro ser leu tyr ser ile val ser pro phe ile pro leu leu pro ile phe phe cys leu trp val tyr ile OC
AUG UGG UAU UGG GGG CCA AGU CUG UAC AGC AUC GUG AGU CCC UUU AUA CCG CUG UUA CCA AUU UUC UUU UGU CUC UGG GUA UAC AUU UAA

ACC CUA ACA AAA CAA AAA GAU GGG GUU AUU CCC UAA ACU UCA UGG CCU ACA UAA UUG GAA GUU GGG GAA CUU UGC CAC AGG AUC
```

Fig. 4 The amino acid sequence of the HBV surface antigen gene. The nucleotide sequence presented in Fig. 3 was translated in one reading frame so as to correspond to the known first 19 amino acids of the N-terminus of the HBV surface antigen[13].

definitive assignment can be made.

It is possible that a putative precursor could extend from the N-terminus. In certain instances, promoter sites in eukaryotic genes may be identified from the DNA sequence. Hogness and Goldberg (personal communication) have postulated that the canonical sequence TATAAATA may interact with RNA polymerase, as *E. coli* polymerase interacts with the Pribnow box[27]. Initiation of transcription usually occurs 23 ± 1 nucleotides from this site. We have been unable to find this sequence in the 200 nucleotides immediately preceding the N-terminal methionine. However, more distant variations on the Hogness–Goldberg sequence occur (D. S. Hogness, personal communication; E. Ziff, personal communication and ref. 28). For example, the sequence TATAIT is found 184 bases in the 5′ RNA direction from the initiator methionine codon of the surface antigen gene and also occurs in the IgG2 light chain[29]. However, there are no specific data and the site of initiation of transcription and the structure of the initial gene product of the HBV surface antigen gene therefore remain in doubt.

Tiollais and coworkers have defined the single-stranded regions in the DNA of the virus[20]. From their map, it is clear that single-stranded regions of DNA extend into the surface antigen gene, but probably not as far as the initiation of transcription. These observations localise the coding strand of the gene on the full-length DNA strand.

The availability of abundant quantities of hepatitis B viral DNA through molecular cloning procedures will allow a comprehensive study of its gene products and the pathology of the virus. The present work enables specific studies on the expression of the gene coding for the major surface antigen to be carried out. The availability of this gene may also provide an alternative means for production of the antigen which in turn can be used as a vaccine against hepatitis B infection.

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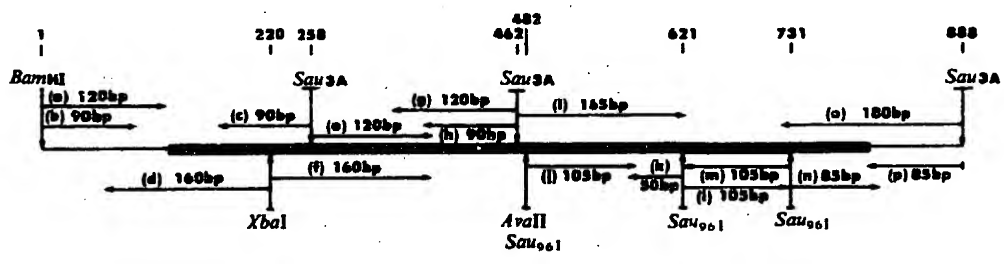

Fig. 5 Sequence strategy for the 889-base pair fragment containing the HBV surface antigen gene. Nucleotide residues are numbered in the direction from 5′ to 3′ in the message strand, beginning at the *Bam*HI site. The solid bar represents the 678-base pair region coding for the protein. Only the restriction sites used as starting points for sequencing are shown. For labelling, DNA was treated with 5 μg of bacterial alkaline phosphatase (Worthington) at 37 °C for 60 min in 20 mM Tris-HCl (pH 8.0), phenol extracted and treated with 10 units of T4 polynucleotide kinase (Boehringer) at 37 °C for 30 min in a reaction mixture containing 50 mM glycine (pH 9.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM spermidine and 0.001 mM [γ-^{32}P]ATP (~4,000 Ci $mmol^{-1}$). Fragments labelled only at one end were isolated by gel electrophoresis after either digestion with a restriction enzyme or strand separation. DNA sequence was carried out by the method of Maxam and Gilbert[34]. Arrows indicate the direction and number of base pairs sequenced in each experiment. Sequences were determined in both strands or in duplicate for all but 50 residues. In this 50-base pair region the data were very reliable. (Sequence data are available from the authors on request.)

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letters

Discovery of IR bursts from Liller I/MXB1730−333

THE first detection of IR bursts from the object[1] known as Liller I which has been identified[2] with the rapid X-ray burster MXB1730−333 is reported here. The rapid burster is the only one of its kind that has been extensively studied in the X-ray region[3-5] and some of its known characteristics can be summarised as follows. When it is active it gives several thousand X-ray bursts per day. It operates in two modes: mode I (usually in March and September every year), mode II (usually in April and October every year). In mode I it gives large X-ray bursts with energy in the range 10^{39}–10^{40} erg and also small bursts with energy in the range of 10^{38}–10^{39} erg; while in mode II it primarily emits bursts with energy typically close to 10^{39} erg. These bursts are called type II on the basis of the constant character of their spectrum during the decay phase. The rapid burster occasionally also gives bursts known as type I which are characterised by the softening of the X-ray spectrum during the decay phase. The energy in these bursts is usually in excess of 10^{39} erg and their frequency is about one every few hours.

Liller[1] searched for an optical counterpart of the rapid burster and found a highly reddened compact cluster with a red magnitude $m_r = 21$ in the error box of the rapid burster[6]. This has been identified as a globular cluster by Kleinmann *et al.*[7], with the help of IR observations. The identification of this cluster with the rapid burster was supported by Doxsey *et al.*[2] who obtained a more precise position for the X-ray burster. Kleinmann *et al.*[7] observed the globular cluster continuously for about an hour on 29 May 1976 and did not detect any variation greater than 10% of the total IR flux. However, the rapid burster may have ceased activity in April 1976.

Two of us (K.M.V.A. and S.M.C.)[8] have proposed a model for the X-ray emission from the rapid burster based on accretion of matter onto the poles of a rotating magnetic neutron star. More recently (unpublished data), the cyclotron emission of electrons in the accreting column above the poles of the magnetised neutron star has been considered and emission in the IR and optical regions suggested; these calculations have prompted us to undertake the present experiment. The optical emission from the rapid burster may, however, be difficult to detect because of the large interstellar extinction[1,7] of $A_v = 11 \pm 1$ mag. On the other hand, the interstellar extinction in the IR is very small which makes it possible to observe the radiation.

The observations were made on the 1-m telescope of the Indian Institute of Astrophysics at Kavalur (lat. 12° N), India on the night of 4–5 April 1979 between 21.29 and 00.10 UT. At the Cassegrain focus a liquid-nitrogen cooled photometer was used with a set of IR filters, three apertures and a Fabry mirror imaging the primary of the telescope on a 0.5 mm × 0.5 mm InSb detector. The incoming beam was $f/20$ giving the scale of 10 arc s mm^{-1} at the focal plane. For the present measurement we used a 2 mm aperture corresponding to 20 arc s field of view. The incoming beam was chopped alternately on the source and a neighbouring part of the sky with a throw of 20 arc s by a tertiary mirror inclined at 45° to the incident beam with a frequency of 16 Hz. The servo-controlled square wave gave a duty cycle of 75%. The Liller I signal was detected with the phase sensitive detector followed by the low pass filter with a time constant of 0.6 s. In the case of standard stars, the phase sensitive detector

Table 1 IR bursts from Liller I/MXB1730−333

Burst No.	Time of occurrence (UT)			Rise time (s)	Duration (s)	FWHM (s)	Peak luminosity (10^{37} erg s^{-1} in 0.3 μm interval at 1.6 μm)	Burst energy (10^{38} erg in 0.3 μm interval at 1.6 μm)
1	21 h	33 min	00 s	2	37	12	2.2	3.0
2	22	41	24	2	36	12	1.9	2.0
3	23	45	06	3	36	12	2.0	2.6
4	23	47	30	3	24	9	1.7	1.5
5	00	03	00	2	36	10	2.2	2.5
6a	00	04	54	2	20	6	2.3	1.7
6b	00	05	12	2	19	6	1.6	0.9

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Biochemistry

Multiple chemical forms of hepatitis B surface antigen produced in yeast

(protein structure/disulfide bonds/vaccine/thiocyanate)

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Contributed by Edward M. Scolnick, June 28, 1985

ABSTRACT Hepatitis B surface antigen (HBsAg) has been extracted from yeast cells that produce HBsAg. These cells contain the gene for surface antigen carried on a plasmid that replicates in the cells. Analysis of the yeast-derived HBsAg by sucrose gradient centrifugation and by polyacrylamide gel electrophoresis shows that the antigen that is initially released from yeast cells is a high molecular weight aggregate of the fundamental M_r 25,000 subunit. Unlike HBsAg derived from human plasma, the yeast antigen is held together by noncovalent interactions and can be dissociated in 2% $NaDodSO_4$ without the use of reducing agents. During *in vitro* purification of the yeast antigen, some disulfide bonds form spontaneously between the antigen subunits, resulting in a particle composed of a mixture of monomers and disulfide-bonded dimers. Treatment with 3 M thiocyanate converts the 20-nm particles into a fully disulfide-bonded form that is not disrupted in $NaDodSO_4$ unless a reducing agent is added. This disulfide-bonded particle resembles the naturally occurring, plasma-derived surface antigen particle, and the *in vitro* formed particle has been used to prepare a vaccine for humans against hepatitis B virus infection.

Hepatitis B surface antigen (HBsAg) derived from human blood is a complex particle, about 20 nm in diameter, composed of protein, carbohydrate, and lipid (1, 2). The protein portion, which carries the antigenic determinants (3), is composed of two types of subunits that share a common polypeptide sequence but that occur either as a nonglycosylated, M_r 25,000 molecule or as a glycosylated, M_r 28,000 form (4–6). In the HBsAg particle these polypeptides are joined by disulfide linkage into dimers and higher multimers. Purified, formaldehyde-treated antigen, isolated from human plasma, is an effective vaccine for the prevention of hepatitis B infection (7). Recently, the HBsAg gene has been cloned in yeast (8–11) and a vaccine has been made that is safe and immunogenic in humans (12, 13) and is effective in preventing hepatitis B infection in chimpanzees (14).

The production of useful proteins by recombinant DNA techniques requires not only that the correct polypeptide be expressed but also that the polypeptide assume the correct three-dimensional structure. For multimeric proteins such as insulin, immunoglobulin, and HBsAg, tertiary structure is maintained by intrachain and interchain disulfide bonds.

Although the HBsAg particle purified from yeast has the form of a 20-nm particle (14–16), it has not been clear whether this structure is assembled in the yeast cell or is a product of the purification process. In addition, it was not known whether the interchain and intrachain disulfide bonds were formed in the cell or after extraction.

In this communication we present evidence that HBsAg produced in yeast is initially released as a large particle in which the subunits are held together by noncovalent interactions (form I). *In vitro*, this form can be converted to a second form in which individual polypeptides are joined together by disulfide bonds into dimers (form II). Finally, disulfide bonds may be formed *in vitro* between dimers to create a disulfide-linked particle (form III) that is similar to the plasma-derived antigen in appearance and chemical and immunological properties.

MATERIALS AND METHODS

Source of Antigen. Human plasma antigen was isolated by the procedure that is used to manufacture vaccine (17). Antigen was taken before the addition of formaldehyde.

Two yeast strains were used as a source of HBsAg. The first is that described by Valenzuela *et al.* (15). The second used a glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter instead of the alcohol dehydrogenase (ADH) promoter. Cells were grown as described by Carty *et al.*‡ and harvested by continuous-flow centrifugation through a Sharples AS16 centrifuge (10.2-cm bowl: flow rate, 3 liters/min). The cell paste (3–5 kg) was suspended in an equal volume of hypertonic phosphate buffer (0.1 M sodium phosphate, pH 7.2/0.5 M NaCl). Phenylmethylsulfonyl fluoride (0.2 M in isopropanol) was added to a final concentration of 2 mM and the cells were disrupted by seven or nine passes through a high-pressure homogenizer (Gaulin, Everett, MA).

Antigen Purification. The crude extract (32–70 mg of protein per ml) was diluted with 4 vol of 0.01 M phosphate buffer (pH 7.5) containing 0.1% Triton X-100 (Rohm and Haas). Cell debris was removed by continuous-flow centrifugation as before, and the supernatant solution was concentrated 5-fold in a hollow fiber unit (H10X100, Amicon) and diafiltered with 2 vol of phosphate-buffered saline [P_i/NaCl (7 mM sodium phosphate, pH 7.2/0.15 M NaCl)]. Triton X-100 was removed by using XAD-2 beads (Rohm and Haas; see ref. 18) and the solution was clarified by centrifugation for 35 min at 9000 × *g* and 4°C. HBsAg was partially purified by adsorption and elution from fused silica (Aerosil 380, Degussa, Teterboro, NJ; see ref. 19).

Partially purified antigen, processed through the silica treatment step, was routinely stored at −70°C. When these samples were thawed, an insoluble precipitate formed. The precipitate was removed by centrifugation at 8000 × *g* for 45 min and the resulting clarified product was purified by hydrophobic chromatography on butyl-agarose (Miles–Yeda, Rehovot, Israel) (unpublished data). Alternatively, the crude yeast cell extract was clarified by Sharples centrifugation and purified by immunoaffinity chromatography as described (20).

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Abbreviations: ADH, alcohol dehydrogenase; GAP, glyceraldehyde-3-phosphate dehydrogenase; HBsAg, hepatitis B surface antigen.

‡Carty, C. E., Kovach, F. X., McAleer, W. J. & Maigetter, R. Z., Eighty-Fourth ASM Meeting, March, 12, 1984, St. Louis, MO, abstr. 030, p. 194.

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Antigen concentration was measured by using a commercial RIA kit (AUSRIA, Abbott). Protein was measured by the Lowry method (21).

PAGE. Antigen samples were disrupted by heating at 100°C for 15 min under nonreducing conditions (2% $NaDodSO_4$/62 mM Tris·HCl, pH 6.8/10% glycerol/6 ppm of bromphenol blue) or under reducing conditions (10 mM dithiothreitol added) as indicated and were separated by electrophoresis through 12.5% acrylamide slab gels as described by Laemmli (22). After electrophoresis, HBsAg polypeptides were visualized by an immunological protein blotting method similar to that described by Burnette (23). Transfer to nitrocellulose sheets (Millipore type HAHY, 0.45 μm) was done in 25 mM sodium phosphate (pH 6.5) for 90 min at 1.5 A. After transfer, the nitrocellulose was saturated by incubation for 2 hr at room temperature in a solution containing 20% gamma globulin-free calf serum (GIBCO), 0.15 M NaCl, 50 mM Tris·HCl (pH 7.6), and 0.1% NaN_3 and then incubated for 90 min with antiserum from rabbits that had been immunized with reduced and denatured plasma-derived HBsAg. Following incubation with ^{125}I-labeled protein A (70–100 μCi/μg; 1 Ci = 37 GBq; New England Nuclear) the blots were exposed to Kodak SB5 x-ray film held between two Cronex Lightning Plus intensifying screens (DuPont).

RESULTS

Although it was known that HBsAg purified from yeast is an aggregate of M_r 25,000 subunits, it was not clear whether these aggregates were present in crude extracts or were formed during purification. To distinguish between these two possibilities, a sample of yeast extract was subjected to velocity sedimentation through a sucrose gradient. Fractions were analyzed for HBsAg by RIA and the sucrose concentration was measured by refractometry. Plasma-derived HBsAg was centrifuged in a companion tube (data not shown). Both the plasma- and yeast-derived antigens sedimented to a density of 25–35% sucrose. Although the position in the sucrose gradient (Fig. 1*A*) indicates that the yeast-derived HBsAg is a high molecular weight particle, $NaDodSO_4$/PAGE analysis under nonreducing conditions (Fig. 1*B*) shows the particles to be composed of M_r 23,000 monomers and M_r 37,000 dimers. $NaDodSO_4$/PAGE analysis under reducing conditions (data not shown) revealed only monomers, indicating that the dimers were held together by disulfide bonds.

Purification by treatment with silica and chromatography over butyl-agarose did not appreciably change the covalent structure of the antigen since most of the immunoreactive polypeptide continued to migrate as monomer and dimer (Fig. 2, compare lanes 7, 8, and 9). By contrast, HBsAg purified from yeast by immunoaffinity chromatography did not enter the running gel under these conditions (Fig. 2, lane 3).

Since purification by immunoaffinity chromatography (which uses 3 M ammonium thiocyanate to elute the antigen) resulted in disulfide-bonded particles, whereas purification by butyl-agarose chromatography yielded non-disulfide-bonded particles, we investigated the possibility that the conversion to disulfide-bonded particles is catalyzed by thiocyanate. $NaDodSO_4$/PAGE analysis of the clarified yeast extract showed a major band of antigen at M_r 24,000, whether or not a disulfide reducing agent was used (Fig. 3, lanes 1). When this clarified extract was treated with 3 M ammonium thiocyanate for 16 hr at 5°C, the antigen no longer entered the running gel unless it had been reduced (Fig. 3, lanes 2). During the preparation of the silica-treated product, part of the antigen was converted to a form that migrated as a mixture of monomer and dimer under nonreducing conditions but still migrated as a monomer when 10 mM dithiothreitol was included in the sample disruption buffer (Fig. 3, lanes 3).

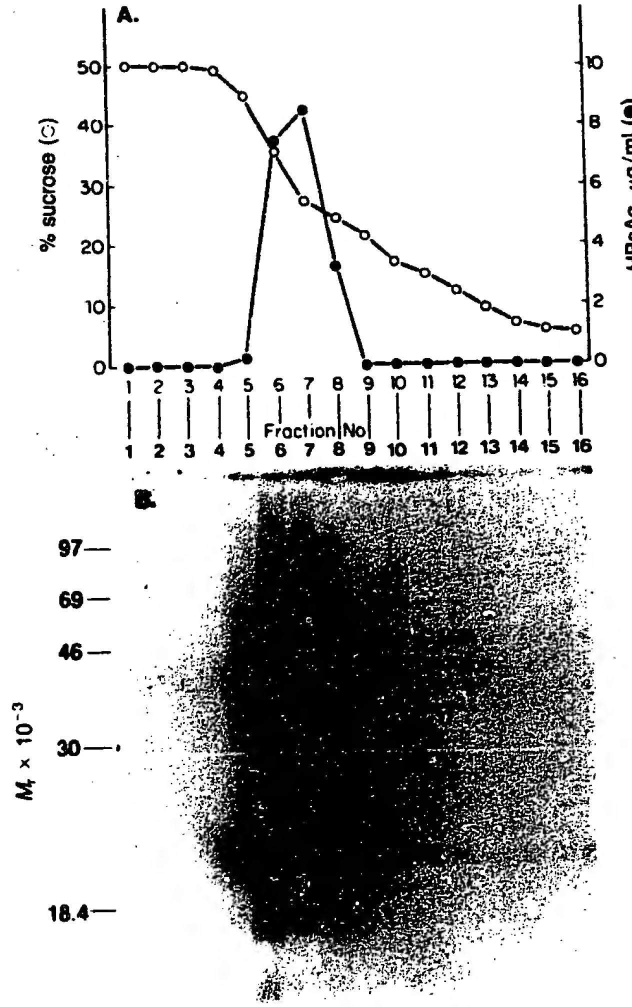

FIG. 1. Sucrose gradient centrifugation of a clarified yeast extract. A 5-ml portion of yeast extract (GAP promoter) was clarified by centrifugation for 15 min at 10,000 × *g*. One milliliter (30 mg of protein) of the clear solution between the pellet and the fatty layer was applied to a 35-ml sucrose gradient (5–50% sucrose), overlayered with 2 ml of P_i/NaCl, and centrifuged for 18 hr at 19,000 rpm (5°C) in a Beckman SW28 rotor. Fractions (2.5 ml) were collected from the bottom of the tube. Each fraction was tested for sucrose concentration by refractive index and for HBsAg concentration by RIA and by nonreducing $NaDodSO_4$/PAGE. (*A*) Elution position in the sucrose gradient. ○, Sucrose concentration; ●, HBsAg concentration measured by RIA. (*B*) $NaDodSO_4$/PAGE under nonreducing conditions. A 25-μl sample of the indicated fraction was mixed with an equal volume of disruption buffer and heated at 100°C for 15 min. Half of the disrupted sample was applied to a slab gel composed of a 3% acrylamide stacking gel and a 12.5% running gel. Separated polypeptides were transferred to nitrocellulose, allowed to react with rabbit antiserum against denatured human plasma HBsAg, followed by ^{125}I-labeled protein A, and visualized by autoradiography. ^{14}C-labeled molecular weight standards include phosphorylase b (M_r 97,000), bovine serum albumin (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), and lactoglobulin A (M_r 18,400).

Additional evidence for the role of thiocyanate in the conversion is given by the concentration dependence shown in Fig. 4. Antigen purified by butyl-agarose chromatography is composed of a mixture of monomers and dimers (Fig. 4, lane 1). After incubation with 1.5 M thiocyanate a majority of the antigen still migrated into the running gel (Fig. 4, compare lanes 1 and 2). After treatment with 3 M thiocyanate the conversion was complete (Fig. 4, lane 3) and essentially all of the antigen was excluded from the running gel.

Fig. 4 also shows that the conversion can be affected by

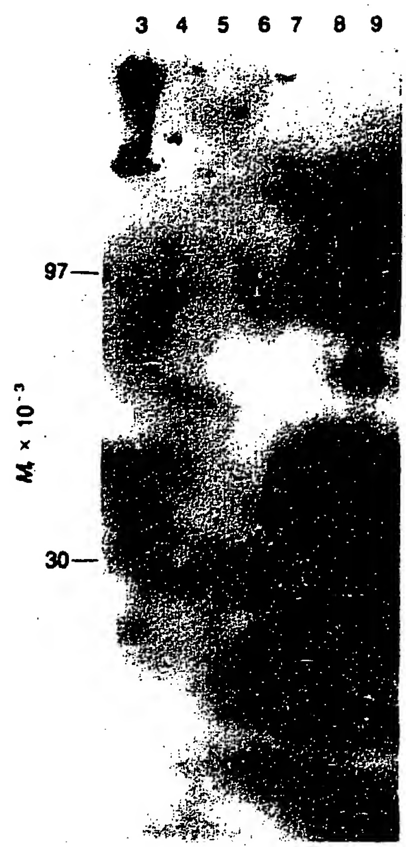

FIG. 2. Forms of HBsAg isolated from yeast cells as a function of the purification process. Three cell extracts were prepared. The first extract (ADH promoter) was purified by immunoaffinity chromatography, with 3 M KSCN as the eluting solvent, as described (20). The second extract (GAP promoter) was purified by the silica treatment, butyl-agarose chromatography process. A third extract, one of the parent yeast strains that does not contain the HBsAg gene, was included as a negative control. Each sample was heated (15 min at 100°C) in disruption buffer under nonreducing conditions and analyzed by $NaDodSO_4$/PAGE. After electrophoresis, samples were transferred to nitrocellulose and HBsAg was detected by immunoblot with rabbit antiserum to denatured human HBsAg followed by reaction with radiolabeled protein A and autoradiography. Lanes 5, 7, and 8 contained 5 μg of total protein; lanes 3 and 9 contained 1 μg of total protein. The lanes were loaded as follows: lane 3, HBsAg from yeast purified by immunoaffinity chromatography; lane 4, empty; lane 5, extract of the parent yeast strain without the HBsAg gene; lane 6, empty; lane 7, clarified yeast extract after treatment with silica; lane 8, clarified silica-treated product; lane 9, butyl-agarose product. Molecular weight standards as in Fig. 1.

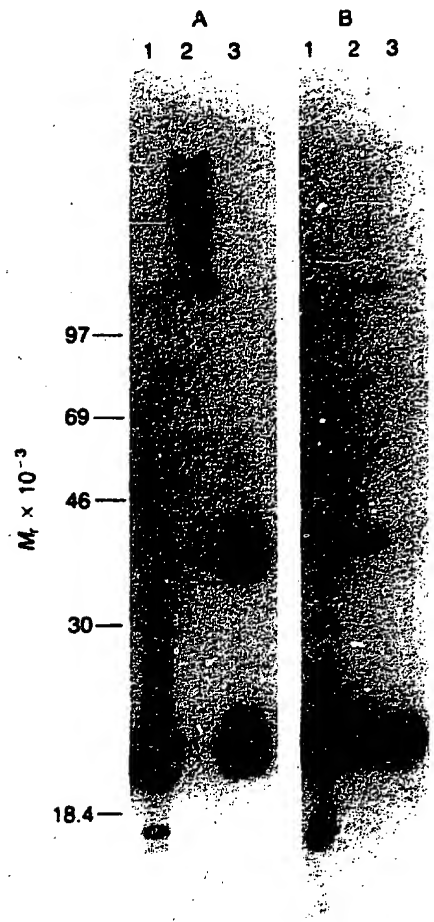

FIG. 3. Formation of intramolecular bonds by 3 M KSCN. A crude yeast extract was prepared from 3.3 kg of yeast cells (ADH promoter). A 100-ml portion of the extract (70 mg of protein per ml) was clarified by centrifugation for 30 min at 36,000 × g. A 5-ml portion of the clarified extract was dialyzed overnight (5°C) against 1 liter of 0.1 M sodium phosphate buffer (pH 7.2) containing 3 M NH_4SCN and 0.5 M NaCl. The precipitate that formed was removed by centrifugation (30 min at 10,000 × g) and the supernatant solution was dialyzed against two 1-liter changes of P_i/NaCl. The remainder of the crude extract (3.7 liters) was processed through the silica treatment step. Samples were heated (15 min at 100°C) in disruption buffer under nonreducing conditions (*A*) or reducing conditions (10 mM dithiothreitol added) (*B*). Disrupted samples were applied to a slab gel composed of a 3% acrylamide stacking gel and a 12.5% running gel. Separated polypeptides were transferred to nitrocellulose, allowed to react with rabbit antiserum against denatured human plasma HBsAg, followed by ^{125}I-labeled protein A, and visualized by autoradiography. Lanes 1, cell extract clarified by centrifugation, 48 μg of total protein; lanes 2, same as lanes 1 after thiocyanate treatment, 45 μg of protein; lanes 3, partially purified antigen without thiocyanate treatment, 3.5 μg of total protein. Molecular weight standards as in Fig. 1.

elevated pH (Fig. 4, lanes 4–7). This approach has the disadvantage, however, that the resulting antigen has lost much of its immunoreactivity. At pH 11.5 and pH 12.5 the antigen lost 29% and 92%, respectively, of its reactivity in RIA.

Thiocyanate might promote interchain disulfide bond formation by facilitating disulfide exchange (intrachain to interchain) within an already oxidized polypeptide. In some cases (24, 25), the chemical- or enzyme-catalyzed disulfide exchange has been shown to be the rate-limiting process in protein renaturation rather than the oxidation itself. Thiocyanate participation in such a reaction might resemble the Na_2SO_3/O_2 sulfitolysis of disulfides to *S*-sulfonated derivatives that can be followed in a second step by thiolysis to reform disulfide bonds (26), a process used in the production of insulin.

To investigate the possibility that thiocyanate was acting as a catalyst for disulfide exchange, two experiments were conducted with low concentrations of thiol reagents. In the first experiment, samples of butyl-agarose-purified antigen were made 0.1 mM or 1 μM in either 2-mercaptoethanol or glutathione. These samples were held for 24 hr at 4°C. The $NaDodSO_4$/PAGE results (not shown) showed no increase in interchain crosslinking. In the second experiment, portions of a clarified extract were treated with mixtures of reduced and oxidized glutathione at a 10:1 ratio (4.5 mM reduced and 0.45 mM oxidized glutathione or 45 μM reduced and 4.5 μM oxidized glutathione). This treatment also failed to increase the extent of interchain disulfide crosslinking.

DISCUSSION

The work reported here shows that the mature form of HBsAg, held together by disulfide bonds, is not present in the initial yeast cell extract but rather must be made outside the cell by some chemical process. After cell lysis, the clarified

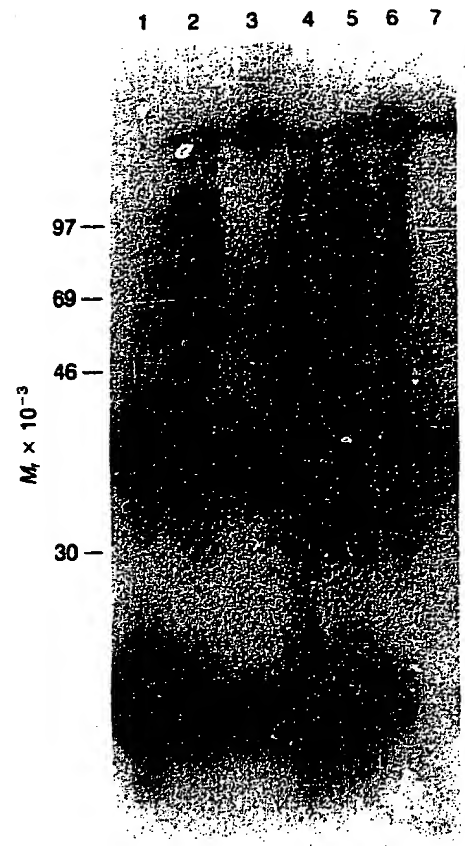

FIG. 4. Conversion to form III at different thiocyanate concentrations or elevated pH. A sample of HBsAg, purified by butyl-agarose chromatography from cells containing the GAP promoter, was divided into four portions. Each portion (0.5–1.5 ml, 0.57 mg of protein per ml) was mixed with an equal volume of P_i/NaCl or of 3 M KSCN (final concentration, 1.5 M) or of 6 M KSCN (final concentration, 3 M). To one of the portions (1.5 ml) that had been mixed with P_i/NaCl, 1 M NaOH was added dropwise, with stirring, and samples were removed at the indicated pH. After 16 hr at room temperature, a 5-μl portion of each sample (2.4 μg of protein) was removed and diluted 1:5 with deionized water. This diluted sample was mixed with an equal volume of disruption buffer and heated with 100°C for 15 min. Half of the disrupted sample was applied to a slab gel composed of a 3% acrylamide stacking gel and a 12.5% running gel. Separated polypeptides were transferred to nitrocellulose, allowed to react with rabbit antiserum against denatured human plasma HBsAg, followed by ^{125}I-labeled protein A, and visualized by autoradiography. Lane 1, control, mixed with P_i/NaCl only; lane 2, treated at 1.5 M thiocyanate; lane 3, treated at 3 M thiocyanate. Samples in lanes 4–7 were treated with NaOH instead of thiocyanate. Lane 4, pH 9.5; lane 5, pH 10.5; lane 6, pH 11.5; lane 7, pH 12. Molecular weight markers as in Fig. 1.

extract contains HBsAg particles that have the same sedimentation coefficient as mature particles but are not held together by covalent bonds. We call this initial particle form I. It is characterized by its migration as a M_r 25,000 monomer in $NaDodSO_4$/PAGE under nonreducing conditions. During early phases of purification, some interchain disulfide bonds form so that the antigen migrates as a mixture of monomer and dimer under nonreducing conditions. We call the disulfide-linked dimers, again recognized in nonreducing $NaDodSO_4$/PAGE, form II. After treatment with concentrated ammonium or potassium thiocyanate, additional interchain disulfide bonds form between dimers, and the fully crosslinked particle is formed. We call this mature particle form III. It is recognized by its inability to enter the running gel of the $NaDodSO_4$/PAGE system unless a reducing agent is used in the disruption step.

It should be emphasized that forms I–III do not represent differences in aggregation state (particle size). Since the $NaDodSO_4$/PAGE procedure uses antibody to fully denatured antigen, it detects all of the HBsAg polypeptide, regardless of conformation or aggregation state. Such $NaDodSO_4$/PAGE analysis of sucrose gradient fractions (Fig. 1) shows no evidence for free monomers. This result agrees with the observations of Valenzuela *et al.* (27), who reported that the antigen travels as a high molecular weight aggregate in size-exclusion chromatography. On the other hand, Heitzeman *et al.* (15) reported that only a small fraction of the HBsAg in yeast cells is aggregated into particles. Their conclusion was based on a comparison of autoradiograms with RIA titers. The difference between these two estimates of HBsAg content was attributed to the presence of monomers that were detected in the autoradiograms but were nonreactive in the RIA. They did not attempt any physical separation of high molecular weight and the proposed low molecular weight forms.

The primary structure of HBsAg contains 14 cysteines per 226 amino acid monomer (8), providing opportunity for numerous different disulfide bonds. Such disulfide bonds contribute to protein stability and proteins with many disulfide links are often resistant to nonreducing denaturing conditions (28, 29). Disulfide links seem particularly important in the stabilization of structural proteins, cell receptors, and extracellular proteins such as immunoglobulins (29). The high cysteine content of HBsAg may explain its unusual heat stability.

Although the "spontaneous" (i.e., O_2) oxidation of protein thiols can take place extracellularly, intracellular disulfide formation either may not occur at all or may be carried out by a variety of mechanisms, including chemical exchange with a pool of oxidized and reduced glutathione (30), various disulfide interchange enzymes coupled with NADPH-dependent glutathione oxidases (31, 32), or sulfhydryl oxidases (33, 34). Heterologous proteins expressed in *Escherichia coli* may remain in a reduced state or may have incorrectly paired disulfide bridges (28, 35, 36). The problem of forming the "correct" disulfide links becomes even more complicated when interchain disulfide bonds are necessary, as in the formation of active insulin from separate A and B chains (see, for example, ref. 37).

Several methods have been used in other laboratories to facilitate the correct disulfide bond formation (23, 38). Saxena and Wetlaufer (24) showed that a mixture of oxidized and reduced glutathione accelerated the reformation of active lysozyme. Cabilly *et al.* (39) and Boss *et al.* (40) used this glutathione mixture to form functional immunoglobulin from polypeptides synthesized in *E. coli* after denaturing the polypeptides in guanidine and urea and then allowing the disulfide bonds to reform. Our results, which failed to show increased disulfide crosslinking after glutathione treatment, suggest that disulfide exchange may not be the mechanism of interchain bond formation in HBsAg from yeast. Instead, these results favor an oxidative mechanism for the thiocyanate conversion.

The oxidation of albumin sulfhydryls by micromoler concentrations of thiocyanogen (NCS-SCN) and by its hydrolysis product, hypothiocyanite ($^-$OSCN), has been examined by Aune and Thomas (ref. 41; see also ref. 42). It was shown that the resulting protein sulfenyl thiocyanate (R-S-SCN) is in equilibrium with the sulfenic acid (R-S-OH) by hydrolysis and that this sulfenic acid can also react with another thiol to form a disulfide link. In our experiments, small amounts of NCS-SCN could be formed in 3 M thiocyanate ($^-$SCN) exposed to air. The R-S-SCN could also react directly with a second sulfhydryl to form the disulfide bond and release thiocyanate and a proton. The details of thiocyanate participation in form III synthesis are a subject for further investigation.

A number of laboratories, including ours, have shown that breaking disulfide bonds in HBsAg reduces the antigenic activity (43–45). In preparing a vaccine, therefore, we have chosen to ensure that the final product is the disulfide-bonded form III. This form of the recombinant antigen, like the antigen from human plasma, has been shown to elicit antibodies in mice, monkeys, chimpanzees, and humans (12–14, 20) and is protective against the experimental hepatitis B virus infection in chimpanzees (14).

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